University of Alberta

The Effect of Benzodiazepines on GABA$_A$ Receptor Homeostasis

by

Robert Andrew Holt

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The overt effects of Diazepam (Valium®), a commonly used anxiolytic and hypnotic drug, are produced through allosteric modulation of $GABA_A$ receptors. $GABA_A$ receptors are pentameric chloride channels that display extensive structural and functional heterogeneity based on the differential assembly of multiple subunit isoforms, each of which is encoded by a separate gene.

In the present study, exposure of rats to daily doses of 15 mg/Kg diazepam modified $GABA_A$ receptor α3-, α4-, α5-, β1-, γ2- and γ3-subunit steady-state mRNA levels in a time- and brain region-specific manner. Diazepam also modified transcription of the $GABA_A$ receptor γ2-subunit gene in rat brain in a manner consistent with the effects of this drug on γ2-subunit steady-state mRNA levels, suggesting a potential transcriptional basis for diazepam-induced changes in $GABA_A$ receptor steady-state mRNA levels. Further, the benzodiazepine-site binding characteristics of native $GABA_A$ receptors in rat brain were modified by chronic diazepam in a manner consistent with the effects of this drug on $GABA_A$ receptor steady-state mRNA levels. Reduced ^{3}H-flunitrazepam but not ^{3}H-Ro15-4513 binding was observed in cortical membranes derived from rats chronically treated with diazepam, as was an increased ratio of zolpidem displaceable versus zolpidem non-displaceable ^{3}H-flunitrazepam binding. Potentiation of ^{3}H-flunitrazepam binding to rat brain membranes by GABA was reduced after a single diazepam dose, but this effect did not appear to be enhanced by chronic treatment and did not appear to be related to diazepam-induced changes in $GABA_A$ receptor gene expression. Finally, the specific pattern of changes in $GABA_A$ receptor subunit mRNA levels produced by chronic exposure to two subtype-specific benzodiazepine-site ligands, abecarnil and zolpidem, which have lower tolerance and dependence liability than diazepam, was different from the pattern produced by diazepam. The results of the present study are consistent with the hypothesis that benzodiazepine exposure evokes subtype-specific changes the population of $GABA_A$ receptors in rat brain as a homeostatic response.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
β-CCE	ethyl β-carboline-3-carboxylate
β-CCM	methyl β-carboline-3-carboxylate
cDNA	complimentary deoxyribonucleic acid
CTP	cytidine triphosphate
DEPC	diethyl pyrocarbonate
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
dpm	disintegrations per minute
EC_{50}	agonist concentration that produces a half-maximal response
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N'-tetraacetic acid
5-HT	5-hydroxytryptamine; serotonin
GABA	γ-aminobutyric acid
GTP	guanosine triphosphate
HPLC	high pressure liquid chromatography
K_i	inhibition constant; the concentration of competing ligand in a competition assay which would occupy half of the receptors if no radioligand were present
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PTZ	pentylenetetrazole
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
SDS	sodium dodecyl sulfate

Tris	Tris(hydroxymethyl)aminomethane
TTP	thymidine triphosphate
UTP	uridine triphosphate

CHAPTER 1

Introduction

GENERAL OVERVIEW

In 1960, introduction of the benzodiazepine chlordiazepoxide into the clinical arena heralded a significant improvement in the pharmacological treatment of pathological anxiety. Since then many benzodiazepine derivatives such as diazepam (Valium®) have been developed and these drugs have achieved widespread use, principally as anxiolytics and hypnotics. An understanding of the mechanism of action of the benzodiazepines was slow to develop and nearly two decades passed following the introduction of chlordiazepoxide before it became clear that these drugs exerted their therapeutic effects via interaction with a receptor for the inhibitory neurotransmitter GABA (γ-aminobutyric acid). GABA is the principal inhibitory neurotransmitter in the mammalian brain and among the most abundant of all neurotransmitters. Fast GABA responses are mediated by the $GABA_A$ receptors, which are ligand-gated chloride channels, and slower GABA responses are mediated by $GABA_B$ receptors, which are coupled via GTP binding proteins and other second messengers to Ca^{2+} and K^+ channels. The benzodiazepines exert their effects specifically through interaction with the $GABA_A$ receptor; they bind to a distinct site and allosterically enhance activation of the receptor by GABA. There are additional binding sites on the $GABA_A$ receptor for separate classes of allosteric modulators such as the barbiturates and endogenous neurosteroids (see Sieghart, 1995, for a comprehensive review of $GABA_A$ receptor pharmacology).

Molecular biology has recently revealed the remarkable complexity of the $GABA_A$ receptor and has placed this receptor within a ligand-gated ion channel superfamily, which also contains the prototypical nicotinic acetylcholine receptor, the glycine receptor, and the 5HT-3 receptor (see Barnard, 1996, for a review of receptor superfamilies). $GABA_A$ receptors are composed of five subunits assembled around a central anion channel. Their functional characteristics are defined by their precise composition of subunits of which, in mammals, there are at least 15 different isoforms (α1–6, β1–3, γ1–3, δ, ε, π), each encoded by a separate gene. Thus, the number of GABA$_A$ receptor subtypes, (i.e. receptors made up of different combinations of subunits) could in theory be vast. Actual GABA$_A$ receptor diversity is, however, probably significantly restricted in comparison to theoretical diversity, but because the complete subunit composition of even a single native GABA$_A$ receptor subtype is yet to be determined this remains unknown.

In a very broad sense, the purpose of this dissertation is to increase our understanding of the effects of benzodiazepines on their molecular target, the GABA$_A$ receptor. The acute action of benzodiazepines is well understood in terms of facilitation of GABA$_A$ receptor activation by the endogenous neurotransmitter, but the sub-acute effects of these agents on the homeostatic control mechanisms that maintain GABA$_A$ receptor function are not clearly defined. There are many different mechanisms through which the GABA$_A$ receptor could mount a homeostatic response to the presence of a benzodiazepine, including desensitization, uncoupling of the allosteric interaction between the GABA and benzodiazepine binding sites, or modification of the rates of receptor assembly, membrane insertion or degradation. Any of these processes could involve changes in the phosphorylation state of the receptor. When considering potential mechanisms for GABA$_A$ receptor homeostatic control an obvious question presents itself. Why are there so many different GABA$_A$ receptor subunits? Why will not a single type of subunit and a single homomeric receptor suffice? While it is known that different neuronal populations express different receptor subtypes in a developmentally specific manner, subtype multiplicity may also be a means of providing GABA$_A$ receptor plasticity. Different GABA$_A$ receptor subtypes expressed *in vitro* have been shown to vary in their functional and ligand-recognition properties. Benzodiazepine pharmacology is particularly dependent upon GABA$_A$ receptor subunit composition. Therefore, in the brain, remodeling of the population of GABA$_A$ receptor subtypes in response to benzodiazepine stimulation may provide a means of adaptation to the presence of the drug. The principal aim of the research described herein is to address the specific question of whether exposure to the classical benzodiazepine diazepam can evoke changes in $GABA_A$ receptor expression in rat brain in a subtype-specific manner, and in a manner which could confer insensitivity to this drug. It is not possible with current technology to identify unambiguously the subunit composition of $GABA_A$ receptor subtypes directly. Therefore, the abundance of different $GABA_A$ receptor subtypes in rat brain, before and after diazepam exposure, has been inferred from the steady-state mRNA levels of various $GABA_A$ receptor subunit isoforms. For example, if in response to diazepam an increase in the steady-state level of α1-subunit mRNA and a decrease in the steady-state level of α2-subunit mRNA are observed, then the proportion of $GABA_A$ receptor subtypes that contain the α1 subunit should increase, while the proportion of α2 subunit-containing receptor subtypes should decline. In the present study predictions of this nature have been tested by exploring the benzodiazepine-site binding characteristics of native receptors, before and after benzodiazepine treatment, using benzodiazepine-site ligands with defined subtype selectivity. The question of whether changes in $GABA_A$ receptor steady-state mRNA levels may have a transcriptional basis has also been addressed.

The clinical implications of this research relate to the well-known phenomena of benzodiazepine tolerance and physical dependence. Prolonged use of therapeutic doses of benzodiazepines can produce physical dependence in a subset of patients, characterized by unpleasant withdrawal symptoms upon cessation of treatment, and studies in animals corroborate these clinical observations (Woods *et al.*, 1992). Conceivably, tolerance and physical dependence could be the manifestations of changes in $GABA_A$ receptor subtype expression which occur at the molecular level as a homeostatic response to benzodiazepine exposure. To explore this possibility, $GABA_A$ receptor mRNA levels have been measured in the brains of rats chronically treated with benzodiazepine-site ligands that have different tolerance and dependence liabilities. If these drugs differentially alter $GABA_A$ receptor mRNA levels, then perhaps changes in $GABA_A$ gene expression are related to the development of benzodiazepine tolerance and dependence.

The benzodiazepines: their development and discovery of their mechanism of action

The benzodiazepine story begins in the 1950s when the clinical value of therapeutic agents known as tranquilizers was becoming established, warranting the search for safer and more efficacious agents. A class of organic molecules known as the benzheptoxdiazines were chosen in a fortuitous manner by Leo Sternbach, then working in the laboratories of Hoffmann LaRoche, as the core compounds on which to base the development of new tranquilizers. These compounds provided the possibility of multiple variations and transformations and their structures suggested they might readily lead to biologically active products. The behavioral testing of a large number of reaction products led to initial disappointment and thus postponement of the program in favor of other problems of presumed greater importance. However, in 1957 testing of a benzheptoxdiazine derivative that had been synthesized several years earlier revealed that this compound, a 1,4-benzodiazepine, had very unique pharmacological properties based on various behavioral tests then used for the screening of tranquilizers - it caused sedation and muscle relaxation, it was anticonvulsant, and it had a pronounced taming effect on laboratory animals indicative of tranquilizing or anxiolytic action. In 1960, following further behavioral, toxicological and clinical testing this compound (chlordiazepoxide) was introduced under the trade name Librium®. Modification of the structure of this benzodiazepine prototype has yielded a family of compounds, known as the classical benzodiazepines, which vary mainly in their pharmacokinetic properties and thus their suitability for different therapeutic applications. For example, the triazolo-benzodiazepine triazolam has a short half-life and is suitable as a hypnotic because it will be metabolized during sleep and, therefore, not cause daytime drowsiness. Benzodiazepines which have a long half-life in man, such as diazepam, are more suitable as anxiolytics due to their sustained action. Currently, the benzodiazepines are the most widely prescribed of all psychoactive drugs.

Following their introduction, a search for the site of action of the benzodiazepines was mounted. A key observation was made in 1967 by Schmidt *et al.*, who noted the ability of diazepam to potentiate pre-synaptic inhibition in the cat spinal cord. After the implication of GABA as the mediator of presynaptic inhibition in the spinal cord several years later (see Levy 1977), the effect of benzodiazepines on synaptic inhibition was re-investigated and it was shown that these drugs enhanced GABAergic transmission. They did not have direct GABA-mimetic action, but the presence of GABA was required for their effect (Polc *et al.*, 1974; Polc and Haefely, 1976). The next crucial step in elucidation of the mechanism of benzodiazepine action was identification, in 1977, of specific high affinity binding sites for ^{3}H-diazepam in synaptic membranes prepared from rat brain (Squires and Braestrup, 1977; Mohler and Okada, 1977). The diazepam receptor identified in these studies appeared to be physiologically significant because strong correlations were observed between the affinity of different benzodiazepines, measured in terms of their ability to displace ^{3}H-diazepam, and pharmacological potency. Benzodiazepines were shown not to act directly at the GABA binding site because known GABA agonists and antagonists failed to influence ^{3}H-diazepam binding, but the association between benzodiazepine action and GABAergic transmission was reinforced by the fact that the rank order of the density of ^{3}H-diazepam receptors in different brain regions was mirrored more closely by that of the GABA receptor than other neurotransmitter receptors.

The realization that the benzodiazepines and GABA interacted with the same physical structure, which we now recognize as the GABA$_A$ receptor, came from several lines of evidence. Shortly after the discovery of the benzodiazepine binding site it was observed that GABA and/or GABA agonists could enhance the binding of radiolabeled benzodiazepines *in vitro* and that this effect could be blocked by the GABA antagonist bicuculline (Martin and Candy, 1978; Tallman *et al.*, 1978; Briley and Langer, 1978), which suggested that even though the GABA and benzodiazepine binding sites were distinct, they were closely coupled. Further, the distributions of binding sites for benzodiazepines and the $GABA_A$ receptor agonist muscimol in rat brain, determined autoradiographically, were markedly similar (Young and Kuhar., 1979; Penney *et al.*, 1981). Finally, isolation of the $GABA_A$ receptor, using a procedure that involved solubilization of receptors from bovine brain and purification via benzodiazepine affinity chromatography, was shown to result in co-purification of high affinity binding sites for GABA and for benzodiazepines, thereby confirming the presence of benzodiazepine binding sites on $GABA_A$ receptors (Sigel *et al.*, 1983).

The precise manner in which diazepam exerts its modulatory effect on $GABA_A$ receptors has been revealed by electrophysiological studies. Initially, it was found that diazepam could modify the kinetic activity of the intrinsic chloride channel of the GABA receptor. In contrast to pentobarbital, a barbiturate, which was found to prolong the length of time the channel remained open in the presence of GABA, diazepam specifically increased the frequency GABA-mediated channel opening events (Study and Barker, 1981). Very recently it has been discovered that in addition to its effects on channel kinetics, diazepam can increase the conductance of $GABA_A$ receptor channels that are activated by low concentrations of GABA, an effect which is greatest in channels with the lowest initial conductance (Eghbali *et al.*, 1997).

Structural and functional characteristics of the $\mathrm{GABA_A}$ receptor

The role of GABA as an inhibitory neurotransmitter was established following its identification (Bazemore *et al.*, 1957) as the principal component of an extract of mammalian brain and spinal cord tissue, termed Factor 1, which was known to inhibit the generation of impulses in the crayfish stretch receptor neuron (Florey, 1954). Subsequent experiments in crustaceans showed that GABA and its biosynthetic enzyme glutamic acid decarboxylase were present specifically in inhibitory axons (Kravitz *et al.*, 1963) and that stimulation of these inhibitory neurons caused GABA to be released (Otsuka *et al.*, 1966). Although the presence of GABA in mouse brain was initially reported in 1950 by Awapara *et al.*, its role as an inhibitory neurotransmitter in the mammalian central nervous system was not confirmed until the late 1960s when it was shown that iontophoretically applied GABA could mimic inhibitory synaptic activity in cat cortical neurons (Krnjevic and Schwartz, 1967; Dreifuss *et al.*, 1969).

The inhibitory actions of GABA in the mammalian brain were initially thought to be mediated by a single class of receptors which upon activation by GABA or muscimol cause a rapid increase in the neuronal membrane permeability to chloride, an effect which can be antagonized by picrotoxin (Takeuchi and Takeuchi, 1969) or bicuculline (Nistri and Constanti, 1979). In fact, these are the receptors we currently know as $\mathrm{GABA_A}$ receptors. The present nomenclature arose following the discovery of a distinct set of GABA receptors in mammalian brain, termed $\mathrm{GABA_B}$ receptors (Hill and Bowery, 1981), which were activated by baclofen but were insensitive to recognized GABA receptor agonists and antagonists. $\mathrm{GABA_B}$ receptors have been identified as members of a superfamily of receptors that exert their cellular effects via coupling to GTP-binding proteins, and have recently been cloned (Kaupmann *et al.*, 1997).

It was the search for the site of action of the benzodiazepines which led to the initial isolation and characterization of the $GABA_A$ receptor. Purified receptors (see Stephenson, 1988 for review) had the same functional characteristics and benzodiazepine, GABA and muscimol binding properties as receptors in their native membrane environment. SDS-polyacrylamide gel electrophoresis indicated the presence of two major proteins of approximately 53 kDa and 57 kDa which were named the α and β subunits, respectively. Based on observations of this nature, and considering the molecular weight of the receptor complex was predicted to be 220 kDa to 240 kDa (Mamalaki *et al.*, 1989), the $GABA_A$ receptor was initially thought to be a homotetramer with $\alpha_2\beta_2$ stoichiometry. However, recent electron microscope images indicate that the receptor, like the nicotinic acetylcholine receptor, is most likely to be pentameric (Nayeem *et al.*, 1994).

Molecular cloning of the α and β subunits (Schofield *et al.*, 1987) provided a major advance in our understanding of the $GABA_A$ receptor. Partial amino acid sequences were obtained for these subunits following their purification from bovine brain. Oligonucleotides were constructed based on this amino acid sequence information, and were used to isolate cDNAs containing the entire protein-coding sequence from bovine cortex cDNA libraries. The complete primary amino acid sequences deduced from the isolated cDNAs were 456 (α) and 474 (β) amino acids in length and suggested that these two subunits were structurally very similar. Each subunit had four putative membrane spanning domains, a large extracellular N-terminal domain containing a β-structural loop and potential N-linked glycosylation sites, and a large intracellular loop between hydrophobic domains three and four which for the β subunit contained a consensus cAMP-dependent protein kinase phosphorylation site. The second transmembrane domain of each subunit was predicted to be the region that lined the receptor's intrinsic chloride channel based on the positions of hydrophilic serine and threonine residues. The overall protein sequence identity between subunits was 35%, but this was much higher in the trans-membrane domains, which were also found to be conserved (approximately 34% identity) with the putative trans-membrane regions of nicotinic acetylcholine receptor subunits. Based on sequence homology and predicted structural similarities between $GABA_A$ receptor and nicotinic acetylcholine receptor subunits, the existence of a ligand gated receptor superfamily was proposed (Schofield *et al.*, 1987).

Neither the early biochemical characterization of the $GABA_A$ receptor nor the initial cloning experiments predicted the heterogeneity of the $GABA_A$ receptor we now recognize. Following identification of the initial α and β subunits by Schofield *et al.* (1987) many other subunits were cloned from various species using various techniques, such that at least 15 different subunits, each encoded by a separate gene, have now been identified in mammals. The architecture of all GABA receptor subunits is conserved, and they have been classified according to nucleotide sequence identity as α1–6, β1–3, γ1–3, δ, ε, π (reviewed by Rabow *et al.*, 1995). Further heterogeneity of the $GABA_A$ receptor is provided by alternate splicing of the α6- (Korpi *et al.*, 1994), β3- (Kirkness and Fraser, 1993) and γ2-subunit (Whiting *et al.*, 1990) transcripts. Thus, the potential exists for a bewildering number of different pentameric $GABA_A$ receptor constructs.

Central to the current hypothesis, which maintains that benzodiazepine exposure causes $GABA_A$ receptor subtype-switching in the brain as a protective mechanism, is the observation that $GABA_A$ receptor subtypes differ in their sensitivity to benzodiazepines and other allosteric modulators. The existence of multiple benzodiazepine binding sites in the mammalian brain was first indicated pharmacologically. Two populations of receptors were differentiated by the triazelopyridazine Cl 218 872 (Squires *et al.*, 1979) and ethyl β-carboline-3-carboxylate (β-CCE) (Nielsen and Braestrup, 1980) which were found to have greater affinity for receptors present in cerebellum than for those in the hippocampus. The cerebellar receptors with high affinity for Cl 218 872 and β-CCE were named BZ1 receptors while the receptors present in hippocampus which had lower affinity for Cl 218 872 and β-CCE were named BZII receptors (Braestrup and Nielsen, 1980).

Molecular cloning of the various GABA receptor subunits has allowed for detailed characterization of the effect of benzodiazepines on $GABA_A$ receptors that are comprised of different combinations of subunits. These experiments have revealed that the presence or absence of a particular subunit in a $GABA_A$ receptor construct can markedly change its sensitive to benzodiazepine modulation. Thus, the complexity of benzodiazepine/$GABA_A$ receptor interaction is much greater than the original BZI/BZII concept suggested. Typically, these experiments have involved either direct injection of $GABA_A$ receptor subunit mRNAs into *Xenopus* oocytes followed by electrophysiological characterization or transfection of $GABA_A$ receptor subunit cDNAs into mammalian cells, the latter approach allowing for electophysiological studies while providing sufficient membrane material for radioligand binding experiments. Using radioligand binding techniques, a robust measure of the affinity (K_i) of a benzodiazepine for a given recombinant receptor can be obtained by measuring its ability to displace a second radiolabeled ligand from the benzodiazepine binding site. Using this approach clear differences in the benzodiazepine-site recognition properties of recombinant $GABA_A$ receptors have been revealed. The efficacy of a benzodiazepine in modulating a given recombinant receptor is more dependent upon exact experimental conditions, such as the precise concentrations of both GABA and the benzodiazepine-site ligand under investigation. Receptors with different subunit compositions do appear to vary in the degree to which they are modulated benzodiazepine site ligands although few studies have been carried out in sufficient detail to clearly define efficacy at distinct receptor constructs. Experiments comparing the potency and efficacy of GABA at recombinant receptors have not indicated any clear and consistent differences conferred by the presence or absence of different subunits.

The properties conferred by different subunit classes, and the various isoforms within those classes, are discussed below. While GABA-gated currents have been reported in both homomeric receptors (Pritchett *et al.*, 1988) and in those comprised of only two different subunit isoforms (Schofield *et al.*, 1987; Levitan *et al.*, 1988), consistent benzodiazepine modulation only occurs in receptors which contain an α, β and γ subunit (Pritchett *et al.*, 1989a; Im *et al.*, 1993; Ymer *et al.*, 1990).

Benzodiazepine modulation of α variant GABA$_A$ receptors

In vitro expression of recombinant GABA$_A$ receptors has indicated that the α subunit isoforms are particularly important in defining the ligand recognition properties of the benzodiazepine site. This was first shown by Pritchet *et al.* (1989b) using HEK (human embryonic kidney) cells co-transfected with β1-, γ2- and various α-subunit cDNAs. In this system ligands used to pharmacologically define BZI and BZII receptors, namely Cl 218 872 and β-CCM (methyl β-carboline-3-carboxylate), showed selectivity for α1 versus α2 or α3 subunit-containing receptors. In contrast, ligands such as diazepam which do not differentiate pharmacologically between type BZI and type BZII binding sites in brain had approximately equal affinity for receptors containing an α1, α2 or α3 subunit. Thus, these experiments defined BZI receptors as sites present on GABA$_A$ receptors which contained the α1 subunit, and BZII receptors as sites present on GABA$_A$ receptors which did not. Further investigation soon showed that benzodiazepine pharmacology could be considerably more diverse than simple BZI/BZII dichotomy. In HEK cells, receptors which contained the α5 subunit plus a γ2 and an arbitrary β subunit showed typical BZII pharmacology, but these constructs were unique in that they had very low affinity for the imidazopyridine compounds alpidem and zolpidem (Pritchet and Seeburg, 1990). Further, receptors expressed in HEK cells containing the α4 or α6 subunit together with a β2 and γ2 subunit were found to be completely devoid of affinity for all benzodiazepine-site agonists tested. These constructs were recognized only by the benzodiazepine-site antagonist flumazenil at moderate affinity and the benzodiazepine-site inverse agonist[1] Ro15-4513 with very high affinity (Luddens *et al.*, 1990; Wisden *et al.*, 1991).

Several studies have shown that modulation of GABA$_A$ receptor chloride conductance by benzodiazepine-site ligands can vary depending on which α subunit isoforms are present. For example, the functional properties conferred by the $\alpha6$ subunit were investigated HEK cells transfected with either the $\alpha_1\beta_2\gamma_2$ or $\alpha_6\beta_2\gamma_2$ combination of cDNAs (Kleingoor *et al.*, 1991). While reduced GABA-activated currents were observed in cells containing either construct in the presence of Ro15-4513, consistent with the inverse agonist activity of this compound, the classical benzodiazepine agonist flunitrazepam potentiated GABA-evoked currents only in cells with the $\alpha_1\beta_2\gamma_2$ combination. Thus, there is agreement between the affinity of these ligands for particular receptor constructs and their modulatory activity. Additional studies have suggested that different α subunit isoforms can confer unique functional responses to benzodiazepine-site ligands in the absence of marked differences in ligand affinity. In HEK cells transfected with either $\alpha1$-, $\alpha2$-, $\alpha3$- or $\alpha5$-subunit cDNA, in combination with $\beta1$- and $\gamma2$-subunit cDNA, maximal potentiation of GABA-evoked currents by diazepam was lowest in cells which contained the $\alpha5$ subunit, and was successively higher in cells containing the $\alpha1$, $\alpha2$ and $\alpha3$ subunits. Likewise, the potency of diazepam was lowest (i.e. the EC$_{50}$ value was highest) in cells which contained the $\alpha5$ subunit (Puia *et al.*, 1991). In *Xenopus* oocytes injected with $\alpha1$ or $\alpha3$, together with $\beta1$ and $\gamma2$ subunit mRNA, flunitrazepam potentiated control GABA currents with a slightly higher potency and maximal efficacy in those oocytes expressing the $\alpha_3\beta_1\gamma_2$ construct (Wafford *et al.*, 1993a), consistent with the responses found in HEK cells.

Differences in functionality conferred by specific α subunit isoforms has also been investigated in terms of coupling of the GABA and benzodiazepine binding sites. In membranes prepared from HEK cells expressing αx, β1 and γ2 subunit-containing receptors GABA potentiated benzodiazepine binding by approximately 200% when the α1 or α2 subunit was present, but approximately 400% when the α3 subunit was present (Pritchet *et al.*, 1989b).

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[1] Benzodiazepine-site inverse agonists, as they have classically been defined, have properties diametrically opposed to those of typical benzodiazepine-site agonists; behaviorally, they are anxiogenic and pro-convulsant and at the molecular level they reduce rather than enhance GABA-mediated chloride curents.

Benzodiazepine modulation of β variant GABA$_A$ receptors

In contrast to the α subunit class, different β subunit isoforms do not appear to markedly influence the affinity of recombinant GABA$_A$ receptors for benzodiazepine-site ligands. Hadingham *et al.* (1993) expressed receptors which contained either an α1, α2 or α5 subunit in combination with a γ2 and one of the β subunits in HEK cells. The exchange of one β subunit isoform for another did not affect the affinity of any of the benzodiazepine-site ligands tested, which included a full range of agonists, inverse agonists, and the antagonist flumazenil. Further, all three β subunits, when expressed in *Xenopus* oocytes together with an α1 and an γ2 subunit isoform conferred equal modulation by benzodiazepines of control GABA responses and no differences were observed in the sensitivity of these β-variant receptors to other allosteric modulators of the GABA$_A$ receptor, including the barbiturate pentobarbital and various neurosteroids. A more recent study has, however, identified an interesting property conferred by a β subunit. Loreclezole (an anticonvulsant that binds to a distinct allosteric site on the GABA$_A$ receptor) has an approximately 300-fold greater affinity for receptors which contain the β1 versus the β2 or β3 subunit (Wingrove *et al.*, 1994).

Benzodiazepine modulation of γ variant GABA$_A$ receptors

The γ subunit class is perhaps the most important in terms of benzodiazepine pharmacology. Recombinant receptors which contain the γ2 subunit are modulated in a robust manner by benzodiazepine-site ligands (Pritchet *et al.*, 1989a) and replacement of the γ2 subunit with another γ subunit isoform gives atypical benzodiazepine pharmacology. Replacement of the γ2 subunit with the γ1 subunit in $\alpha_1\beta_1\gamma_x$, $\alpha_2\beta_1\gamma_x$ or $\alpha_5\beta_1\gamma_x$ receptors expressed in *Xenopus* oocytes had two consequences: the ability of various benzodiazepine-site agonists to potentiate GABA-activated currents was significantly reduced, and the inverse agonists DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) and β-CCM behaved as agonists and actually potentiated GABA responses (Puia *et al.*, 1991; Wafford *et al.*, 1993b). Studies which investigated the binding properties of benzodiazepine-site ligands for receptors comprised of the $\alpha_1\beta_1\gamma_x$ or $\alpha_1\beta_1\gamma_2$ subunit combinations indicated that the affinity of ligands for the benzodiazepine binding site is lower when the γ1 subunit is present, regardless of whether they are positive, neutral or negative modulators (Ymer *et al.*, 1990).

The role of the γ3 subunit in benzodiazepine pharmacology was investigated by Herb *et al.* (1992), and it was shown that this subunit can also confer interesting properties. In co-expression experiments with the α1 and β2 subunits, inclusion of γ3 subunit in place of the γ2 subunit was found to impart markedly lower benzodiazepine site agonist affinity, and also a decreased ability of these drugs to potentiate GABA-activated chloride currents. For example, the affinity of diazepam was 44 times lower for γ3 than γ2 subunit-containing receptors, and, while GABA responses were potentiated approximately 122% at γ2 subunit-containing receptors, potentiation of only 25% was observed with receptors containing the γ3 subunit. In contrast to the marked differences in the sensitivity to benzodiazepine-site agonists conferred by the γ3 subunit, no differences in the affinity or the efficacy of the inverse agonists DMCM and β-CCM were observed between receptors containing the γ2 or γ3 subunit.

The variability in benzodiazepine responses observed in the above γ-variant $GABA_A$ receptors clearly illustrates the limitations associated with the current method of classification of benzodiazepine-site ligands. For example β-CCM, contrary to its classical definition as a benzodiazepine-site inverse agonist, positively modulates chloride currents at γ1 subunit-containing recombinant receptors. Thus, although it may be more cumbersome, a system of nomenclature which accounts for the fact that benzodiazepine-site ligands can have different modulatory effects at different $GABA_A$ receptor subtypes would be more accurate.

$GABA_A$ receptors containing other classes of subunits

In the present study, the homeostatic control of only those $GABA_A$ receptors comprised of α, β and γ subunits is considered. This is primarily because receptors which contain these subunits are the most ubiquitous in the mammalian brain, and heterologously expressed recombinant α/β/γ receptors exhibit the full range of benzodiazepine pharmacology. There are additional $GABA_A$ receptor subunits, some of which have been cloned very recently (Davies *et al.*, 1997; Hedblom and Kirkness, 1997), which tend to be restricted in both their distribution and their pharmacological properties. For example, the π subunit is expressed in the tissues of the reproductive system and its presence in the $\alpha_1\beta_x\gamma_2\pi$ recombinant receptor construct reduces sensitivity to pregnenalone (Hedblom and Kirkness, 1997). The δ (Shivers *et al.*, 1989) and ε (Davies *et al.*, 1997) subunits are present in the mammalian brain, but their distribution is restricted; the δ subunit is expressed mainly in the thalamus and cerebellum (Wisden *et al.*, 1992) and the ε subunit is expressed mainly in the amygdala and subthalamic nuclei (Davies *et al.*, 1997).

Recombinant receptors containing a δ subunit in place of a γ2 subunit are insensitive to benzodiazepines, and those which contain an ε subunit (in $\alpha_2\beta_1\varepsilon$ or $\alpha_1\beta_3\varepsilon$ constructs) are insensitive to modulation by the barbiturates and the anesthetic agent propofol (Davies *et al.*, 1997). Three ρ subunits, which are expressed only in the retina, contribute to a distinct class of receptors called $GABA_c$ receptors that are insensitive to the benzodiazepines, barbiturates, and the GABA antagonist bicuculline (Cutting *et al.*, 1991; Cutting *et al.*, 1992; Shimada *et al.*, 1992; Ogurusu and Shingai, 1996).

The native $GABA_A$ receptor

In spite of the considerable amount of information on $GABA_A$ receptor structure and function that has been obtained through the exhaustive characterization of recombinant receptors expressed *in vitro*, comparatively little is known about the $GABA_A$ receptors that actually exist in the brain. Although some progress has been made in understanding native receptors, the questions (1) which $GABA_A$ receptor subunits actually combine to form functional receptor subtypes *in vivo*, and (2) to what degree are the properties of native $GABA_A$ receptor subtypes predictable based upon the characteristics of recombinant receptors, have not been fully resolved.

Several lines of evidence indicate that certain receptor subtypes are more prevalent in the brain than others. The most abundant receptor subtype, accounting for nearly half of all $GABA_A$ receptors in the brain, is probably that which contains the α1, β2, and γ2 subunits in some combination of five. *In situ* hybridization studies have shown that the mRNAs which encode these subunits are the most widely co-distributed in rat brain and, in addition, there are many regions where only α1-, β2-, and γ2-subunit mRNAs are found (Wisden *et al.*, 1992). These results are corroborated by immunohistochemical studies in which α1-, β2- and γ2-subunit proteins were co-visualized at both the regional and cellular level (Fritschy *et al.*, 1992; Fritschy and Mohler, 1995).

Identifying native GABA$_A$ receptor subtypes is, however, not as easy as simply revealing patterns of co-distribution. *In vitro*, a preference for assembly of recombinant receptors of certain subunit combinations has been indicated (Angelotti *et al.*, 1993), suggesting that the presence of various subunits, even if they are within the same cell, does not guarantee co-assembly. As a more robust approach to identification of native GABA$_A$ receptors, subunit composition has been examined through immunoprecipitation. This approach involves precipitating all receptors containing a certain subunit from brain extract using a subunit-specific antibody, then probing this pool with a second antibody to identify co-assembled subunit pairs. In such experiments the α1, β2, and γ2 subunits have been shown to be the most ubiquitous of their respective classes, and to be preferentially associated with one another (Benke *et al.*, 1994; Quirk *et al*, 1994a,b ; McKernan *et al.*, 1991). Through combinations of the above approaches (in situ hybridization, immunocytochemistry and immunoprecipitation) additional candidates for major native GABA$_A$ receptor subtypes have been proposed, including the $\alpha_2\beta_x\gamma_2$ an $\alpha_5\beta_3\gamma_{2/3}$ combinations on hippocampal pyramidal cells and the $\alpha_6\beta_x\gamma_2$ and $\alpha_6\beta_x\delta$ combinations on cerebellar granule cells (reviewed by McKernan and Whiting, 1996).

Where it has been determined, the pharmacology of native GABA$_A$ receptors is largely consistent with GABA$_A$ receptor pharmacology produced in recombinant systems. The brain areas expressing the highest level of α1-subunit mRNA and lower levels of α2-, α3- and α5-subunit mRNA, such as the cerebellum, are the same areas which show type BZI pharmacology and areas that express relatively little α1-subunit mRNA but higher levels of α2-, α3- and α5-subunit mRNA, such as the caudate-putamen, are enriched in BZII binding sites (Wisden *et al.*, 1992; Young *et al*, 1981). Further, native receptors immunoprecipitated from rat brain whole brain extracts using α1- versus α2-, α3- or α5-subunit antibodies show the appropriate BZI/BZII pharmacology; receptors precipitated using the α1-subunit antibody have higher affinity for CL 218 872 and β-CCM than receptors precipitated with the α2-, α3- or α5-subunit antibodies (McKernan *et al.*, 1991; Benke *et al.*, 1994). Further, native receptors precipitated from rat whole brain extracts using α5-subunit antibody have low affinity for zolpidem (McKernan et al., 1991), though it has been reported that zolpidem's affinity for α5 subunit-containing receptors can vary among brain regions (Mertens *et al.*, 1993). Receptors precipitated from rat cerebellar extracts using an α6-subunit antibody are not bound by classical benzodiazepines such as diazepam and flunitrazepam but maintain high affinity for Ro15-4513 (Quirk *et al.*, 1994a), which is consistent with the pharmacology seen in recombinant systems.

Specific aims

The experiments described in this dissertation have been carried out in order to increase our understanding of the neurochemical changes that occur in association with chronic exposure to benzodiazepines. The principal question that has been addressed is whether exposure to benzodiazepine-site agonists causes $GABA_A$ receptor subtype switching in the brain as a homeostatic response. In each of the following chapters the experiments described were undertaken with a specific aim related to this question. In chapter two, the specific aim was to characterize the effects of the quintessential benzodiazepine diazepam on $GABA_A$ receptor steady-state mRNA levels in rat brain, with the premise that any such changes will modify the $GABA_A$ receptor subtype profile. In chapter three the specific aim was to determine whether diazepam-induced changes in

$GABA_A$ receptor steady-state mRNA levels could potentially involve modification of $GABA_A$ receptor gene transcription and in chapter four the specific aim was to determine whether the effects of diazepam on $GABA_A$ receptor protein, characterized in terms of the recognition properties of the benzodiazepine binding site, coincide with diazepam-induced changes in mRNA levels. In chapter five, the specific aim was to determine whether drugs with dissimilar tolerance and dependence liabilities (diazepam, abecarnil and zolpidem) produce differential changes in $GABA_A$ receptor steady-state mRNA levels.

CHAPTER 2

The effect of diazepam on $GABA_A$ receptor steady-state mRNA levels

Data presented in this chapter have been previously published. Holt, R.A., A.N. Bateson and I.L. Martin, 1996, Neuropharmacol. 35, 1457.

INTRODUCTION

Diazepam is a classical benzodiazepine used extensively as an anxiolytic and hypnotic agent. Like other benzodiazepines, diazepam produces its overt effects by interaction with a specific recognition site on the $GABA_A$ receptor. Considering the multiplicity of $GABA_A$ receptor subunits that can be combined, in various ways, to form a myriad of different pentameric $GABA_A$ receptor subtypes, it is possible that one of the ways in which the GABAergic system homeostatically adjusts to stimulation by diazepam is through modified expression of $GABA_A$ receptor subunit genes, and thus receptor subtypes. This forms an attractive hypothesis for the development of benzodiazepine tolerance and dependence (Heninger *et al.*, 1990) given that receptor subtypes vary in their sensitivity to diazepam and other allosteric modulators. This chapter describes an extensive survey of $GABA_A$ receptor subunit steady-state mRNA levels (taken as an index of $GABA_A$ receptor subunit gene expression) in rat brain, conducted after chronic exposure of the animals to diazepam.

MATERIALS AND METHODS

Drug treatment

All animal procedures described in this dissertation were in accordance with the guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats, 175 g to 200 g at initiation of treatment, were housed two per cage and maintained on a 12 hour light-dark cycle (light 6:00 am to 6:00 pm, dark 6:00 pm to 6:00 am). After a minimum of three days habituation, rats were injected subcutaneously once daily with 15 mg/kg diazepam in 1 ml of sesame oil vehicle for either 7, 14 or 28 days. An additional group of animals was treated for 28 days then subjected to 9 days of drug withdrawal. All vehicle-treated animals were injected subcutaneously with 1 ml of pure sesame oil, and untreated control animals were not injected. Sesame oil was used as a depot to provide sustained drug release and, thus, more sustained levels of receptor occupancy without the need for multiple daily injections (Steppuhn *et al.*, 1993). All rats were killed by decapitation without anaesthetic 2 hours after their final dose, with the exception of the animals comprising the 14 day treatment group, which were killed 24 hours after their last dose[illegible]. Immediately after sacrifice cortex and hippocampus were isolated, frozen in liquid nitrogen and stored at -80°C.

Quantification of CNS diazepam levels by reversed-phase HPLC

In the present study, the brain concentration of diazepam was measured to determine the variation that exists between the animals comprising a given treatment group, and to confirm previous reports that the metabolism of diazepam is not significantly altered by chronic exposure (Haigh *et al.*, 1986; Miller *et al.*, 1988). 100 mg of cortex or spinal

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[2] The diazepam and abecarnil (see chapter 5) 14 day dosing regimens were the first to be completed. Because of difficulties in quantifying drug levels in these animals, which were sacrificed 24 hours after their final dose, animals in subsequent experiments were killed 2 hours after their final dose.

cord was taken from each animal treated with diazepam, homogenized in five volumes of methanol using an Ultra Turrax homogenizer set on medium and centrifuged 15 minutes at 12000 x g, 4°C. The efficiency of this extraction method was 73.4% +/- 3.6% (mean +/- SD). A 50 µl aliquot of the supernatant was injected onto a 30 cm Spherisorb C18 5 µm column (Alltech) and eluted at a flow rate of 1.5 ml/min using a solvent gradient that increased linearly from 65% A:35% B (v/v) to 85% A:15% B (v/v) in 12 minutes, with "A" being methanol and "B" being 13 mM sodium phosphate buffer (pH 7). During the separation, ultraviolet absorption of the eluate was measured at 241 nm. Flunitrazepam (100 ng per 50 µl injection volume) was used as an internal standard.

A standard curve was generated for each assay as follows. Brain extract was prepared from the cortex of an untreated rat and to 50 µl aliquots of this extract 100 ng of flunitrazepam and either 0.2, 1, 2 or 10 ng of diazepam were added. These samples were then analysed as above and the ratio of diazepam peak area to flunitrazepam peak area was plotted against the amount of diazepam present in each sample. By comparison to this plot, which was linear over the range of diazepam concentrations used, the amount of diazepam present in extracts prepared from each drug-treated animal was determined.

The solution hybridization assay - overview

In the present study a multiprobe solution hybridization assay has been used to measure the effect of diazepam on the steady-state levels of specific $GABA_A$ receptor mRNAs in rat brain. This technique was first described by O'Donovan *et al.* (1991) and utilizes short, synthetic, sequence-specific, ^{32}P-labeled oligodeoxyribonucleotide probes which vary in length depending upon which mRNA species they recognize. Probes, present in excess, are incubated with total RNA isolated from brain tissue such that hybridization to every corresponding mRNA molecule is achieved. Following hybridization, unbound probe molecules are removed by digestion with S1 nuclease, an enzyme which preferentially digests single stranded nucleic acids. Probe molecules hybridized to mRNA are protected from digestion and are subsequently resolved using polyacrylamide gel electrophoresis and visualized by autoradiography. Autoradiographic band intensity (quantified using densitometry) is thus a measure of the abundance of specific mRNA molecules initially present, and this value can be compared between samples.

It must be stressed that under normal circumstances $GABA_A$ receptor subunit mRNA species differ markedly in their abundance throughout rat brain (Wisden *et al.*, 1992). Using the solution hybridization assay, no information regarding the absolute quantity of an mRNA species is provided. In the present study each $GABA_A$ receptor subunit mRNA has been quantified in a relative manner by comparing its level in brain tissue taken from drug treated versus control animals. Accordingly, a small percent change in the level of an abundant subunit could be comparable, in absolute terms and in terms of biological relevance, to a large percent change in the level of a rare transcript.

RNA preparation

Total RNA was isolated from cortex or hippocampus using TRIzol reagent (Gibco-BRL) according to the manufacturers instructions. 1 ml of TRIzol reagent was added to 100 mg of brain tissue and immediately homogenized using an Ultra Turrax homogenizer for 30 s at full power. The homogenate was incubated at room temperature for 5 min then 0.2 ml chloroform was added per ml of TRIzol and samples were shaken vigorously for 15s. Samples were centrifuged 15 min at 7500 x g, 4°C, and the aqueous phase was transferred to a new tube. RNA was precipitated by the addition of 0.5 ml isopropanol per ml of TRIzol initially used and incubated at room temperature for 10 min. The precipitate was collected by centrifugation 15 min at 12000 x g, 4°C, washed with 1 ml of 75% ethanol, air dried, resuspended in 100 μl DEPC (diethyl pyrocarbonate) treated water and stored at -80°C. The concentration of isolated RNA was determined by measuring the absorbence of a 1:200 dilution at 260 nm given that 1 A_{260} unit is equivalent to 40 μg/ml RNA.

Oligonucleotide probe synthesis and purification

Oligodeoxyribonucleotide probes complementary to non-conserved regions of rat $GABA_A$ receptor subunit mRNAs and β-actin mRNA were constructed from published cDNA sequences (table 2.1). The γ2-subunit probe does not distinguish between the $\gamma 2_L$- and $\gamma 2_S$- subunit splice variants. Probes were synthesized on an ABI Synthesizer (a service provided by the Department of Biochemistry, The University of Alberta) and purified on denaturing polyacrylamide gels using standard procedures (Ausabel *et al.*, 1994). For purification, a 9% (w/v) acrylamide gel (a 19:1 ratio of acrylamide:bis-acrylamide, 46% (w/v) urea, 89 mM Tris-borate, 89 mM boric acid, 2.5 mM EDTA) was pre-run in running buffer (89 mM Tris-borate, 89 mM boric acid, 2.5mM EDTA) for approximately 45 min, until the temperature of the gel reached 55°C. 40 μg of oligonucleotide in 10 μl of H_20 were added to 10 μl of formamide loading buffer (95% (v/v) de-ionized formamide, 20 mM EDTA, pH 8, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol), denatured 3 min at 95°C and applied to the gel. Following electrophoresis, the gel was transferred to plastic wrap and oligonucleotide bands were visualized by the shadowing of ultraviolet light (254 nm) onto a silica gel thin layer chromatography plate containing zinc silicate fluorescent indicator (Fisher Scientific). The correct, full-length bands were cut out of the gel using sterile scalpel blades and the DNA was extracted by rotating each gel slice overnight in 0.5 ml of 0.3 M sodium acetate. Acrylamide was removed by centrifugation for 5 min at 12000 x g, 4°C, and the oligonucleotide solution was extracted with 0.5 ml of Tris-buffered (pH 7.5) phenol:chloroform:isoamyl alcohol (25:24:1, v/v). The oligonucleotide was precipitated for 30 min, at -20°C, in 2.5 volumes of 95% (v/v) ethanol, recovered by centrifugation for 30 min at 12000 x g, 4°C, and resuspended in 50 µl of DEPC-treated H_2O. The concentration of the purified oligonucleotide stock was determined by measuring the absorbence of a 1:200 dilution at 260 nm, given that 1 A_{260} unit is equivalent to 20 µg/ml of single stranded oligonucleotide.

Oligonucleotide labeling

Oligonucleotides were labeled in separate reactions. 3 pmol of purified oligonucleotide, 50 µCi of 100 mCi/ml, 7000 Ci/mmol γ-^{32}P-ATP (ICN) and 10 U of T4 polynucleotide kinase (Gibco-BRL) were combined in 20 µl (total volume) forward reaction buffer (0.35 M Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 0.5M KCl, 5mM 2-mercaptoethanol; Gibco-BRL) and incubated at 37°C for 45 min. Each reaction was stopped by dilution to 100 µl in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.6) and labeled oligonucleotide was extracted with 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v). The aqueous layer was isolated from the organic layer by centrifugation for 2 min at 12000 x g and then applied to a 1 ml Sephadex G-50 spin-column and centrifuged for 4 min at 2000 x g to remove unincorporated γ-^{32}P-ATP. Each radiolabeled oligonucleotide was then diluted with DEPC-treated H_20 to a final concentration of 0.006 pmol/µl and the specific activity of each stock was determined by liquid scintillation counting. The specific activities of the probes complimentary to the more abundant mRNAs were reduced by dilution with unlabelled probe in order to empirically equalize signal strength and thereby facilitate the simultaneous quantification of abundant and rare mRNAs on the same autoradiogram. For example, stocks of radiolabeled β-actin oligonucleotide were routinely diluted 20x with unlabelled β-actin oligonucleotide stock of equal concentration.

Solution hybridization

In each hybridization experiment up to 7 different mRNA species were simultaneously assayed, including in each case β-actin mRNA, the level of which provided an internal standard to which GABA$_A$ receptor mRNA levels were normalized. The inclusion of an internal standard is necessary to eliminate differences between samples caused, for example, by minor but potentially cumulative variability in pipetting or precipitation/resuspension efficiency.

10 µg of each sample of total mRNA and 0.03 pmol of each labeled oligonucleotide were combined and vacuum-dried. Dried samples were resuspended in 30 µl hybridization buffer (0.4 M NaCl, 40 mM PIPES, pH 6.4, 1mM EDTA) , denatured at 95°C for 3 min and then transferred directly into a 70°C water bath for overnight incubation.

Digestion of excess oligonucleotide probe

Hybridized mRNA and oligonucleotides were removed from the 70°C water bath after overnight incubation and briefly centrifuged. 0.3 ml of ice cold S1 nuclease mix (120 U/ml S1 nuclease (Amersham), 4.5 mM zinc sulphate, 50 mM sodium acetate, pH 4.2, 0.3 M NaCl, 10 µg/ml denatured salmon sperm DNA) was added to each sample and digestion of excess, unhybridized oligonucleotide was allowed to proceed for 15 min at 37°C.

A control sample in which yeast tRNA was substituted for cortex- or hippocampus-derived total RNA was included in each experiment. Because no specific hybridization between tRNA and the labeled oligonucleotides should occur, the absence of bands in the tRNA lane of the autoradiogram indicates complete digestion of unhybridized oligonucleotide.

Following digestion, 0.3 ml of each sample was transferred to a new tube containing 0.75 ml of 95% (v/v) ethanol, precipitated for 30 min at -20°C and centrifuged for 30 min at 12000 x g, 4°C. Pellets were washed with 1 ml of ice-cold 75% (v/v) ethanol, air dried, and resuspended in 6 µl of distilled, de-ionized water.

Separation of protected oligonucleotides by denaturing polyacrylamide gel electrophoresis

A 9% acrylamide gel was pre-run until the gel temperature reached 55°C, as described in the oligonucleotide purification protocol. The 6 µl samples of protected oligonucleotide were combined with 6 µl of formamide loading dye, denatured for 3 min at 90°C, then loaded onto the gel and electrophoresed maintaining a minimum temperature of 55°C until the bromophenol blue dye had migrated approximately 30 cm down the 40 cm gel. Following electrophoresis, gels were allowed to cool below 55°C, the gel plates were separated and an oligonucleotide-impermeable cellophane membrane (BIO-RAD) was applied to one face of the gel in order to prevent oligonucleotide loss during drying. This face of the gel was then transferred onto Whatmann 3MM paper and the other surface of the gel was covered with plastic wrap. Gel sandwiches were dried under vacuum at 80°C for one hour.

Detection of protected oligonucleotides by autoradiography

Dried gels were apposed to BioMax X-ray film (Kodak) in an autoradiography cassette containing an intensifying screen, pre-flashed according to the film manufacturer's instructions and stored for several days at -80°C to allow film exposure.

Densitometric quantification of oligonucleotide band intensities

Developed autoradiograms were imaged concurrently with a gray scale calibration tablet using a flatbed scanner (Hewlett Packard ScanJet 3C). Images were saved in TIFF format and analyzed using NIH Image v1.56 software (Wayne Rasband, RSB, NIMH, NIH, Bethesda, MD) as follows. A gray scale/optical density calibration was performed for each autoradiogram by sampling an area within each step of the co-imaged gray scale calibration tablet, entering the known optical density values of these steps into the NIH Image calibration function, and determining the best-fit calibration curve. The optical density of each lane was plotted (with background being manually subtracted by extending the baseline beneath each peak) and the area of each peak was integrated. After completion of the solution hybridization procedure, the number of protected oligonucleotide probe molecules was proportional to the number of specific mRNA molecules initially present. Therefore, following electrophoresis, autoradiography and densitometric analysis, the number of specific mRNA molecules initially present in a sample is proportional to peak area.

Using autoradiographic techniques, very high band intensity can potentially saturate the detection system (either the X-ray film itself or the image capturing device). Figure 2.1 illustrates the range within which the relationship between the amount of radioactivity contained within bands on an acrylamide gel and the measured optical density of their corresponding autoradiographic bands is linear. Serial dilutions of radiolabeled oligonucleotide were electrophoresed as previously described and the optical density of the resulting autoradiographic bands was plotted against the amount of radioactivity they represented. The linear range of detection indicated was 0 through 5000 optical density units and, accordingly, the exposure time of each autoradiogram containing experimental data was restricted such that band intensity remained within this range.

Linearity and variability of the solution hybridization assay

Using the solution hybridization assay conditions described, autoradiographic band intensity accurately reflects the relative amount of mRNA species under investigation. Figure 2.2 illustrates the linear relationship between increasing amounts of total RNA extracted from rat cortex and the optical density of autoradiographic bands generated following solution hybridization with an oligonucleotide probe specific for β-actin mRNA.

To determine the inter- and intra-assay variability of this technique the relative amount of $GABA_A$ receptor α1-subunit mRNA versus β-actin mRNA in a single preparation of total rat cortex RNA was analyzed three times within a single experiment (i.e. three separate hybridization reactions were resolved in three separate lanes of an acrylamide gel, giving rise to three sets of autoradiographic bands) and then this same sample was analyzed in three separate assays. The inter- and intra-assay indices of variability (standard deviation expressed as a percentage of the mean) were 9.0% and 15.8% respectively.

RESULTS

Diazepam concentrations in rat brain following chronic exposure

The mean concentration of diazepam in rats killed 2 hours after their last of 7 daily injections was 200.3+/-37.7 (mean+/-SEM, n=6) nanograms of diazepam per gram of cortex and in rats treated for 28 days and killed 2 hours after their final dose the concentration of diazepam was 248.3 +/- 98.7 ng/g. These values are not significantly different.

It is not possible to predict, based on the brain concentration of a benzodiazepine-site ligand, the actual number of binding sites occupied. This is due to the fact that the drug will partition between different cellular compartments in the brain and the actual concentration at the synapse will be unknown. However, Steppuhn *et al.* (1993) have shown that a single dose of 15 mg/kg diazepam administered to mice in sesame oil via subcutaneous injection gives levels of benzodiazepines-site occupancy which vary from approximately 80% at 7 hours post injection to approximately 30% at 24 hours post-injection.

The effects of diazepam on GABA$_A$ receptor mRNA levels

No significant changes were observed in the levels of any of the GABA$_A$ receptor subunit mRNA species in cortex or hippocampus, at any of the time points examined, in response to treatment with sesame oil vehicle alone. The abundance of γ1- and γ3-subunit mRNA in hippocampus was too low to allow quantification and, in both the cortex and hippocampus, the level of α6-subunit mRNA was not sufficient for quantification.

Figure 2.3 shows a set of lanes from an autoradiogram and their corresponding density plots, illustrating typical results of the solution hybridization assay. Many significant changes in the levels of specific $GABA_A$ receptor subunit mRNAs in response to diazepam treatment were observed in the present study (table 2.2). In cortex, two weeks of diazepam exposure caused a significant decrease in the amount of $\gamma2$- and a significant increase in the amount of $\alpha3$- and $\alpha5$-subunit mRNA. The levels of $\alpha4$-, $\beta1$- and $\gamma3$-subunit mRNA were significantly elevated after both one and two weeks of diazepam treatment. None of the $GABA_A$ receptor mRNA levels in cortex were significantly different than control values after 28 days of treatment and no significant differences were observed at withdrawal day 9. The effect of diazepam exposure on rat hippocampal $GABA_A$ receptor mRNA levels was, in several instances, different from the effect in cortex. For example, in hippocampus, the levels of $\beta1$- and $\alpha4$-subunit mRNA were not increased until the fourth week of treatment, and the significant changes in $\alpha5$- and $\alpha3$-subunit mRNA observed in cortex did not occur in hippocampus at any time point. Most notably, however, the effect of diazepam on $\gamma2$- subunit mRNA was opposite in these two brain regions. A significant increase in the level of this transcript in hippocampus was observed after both one and two weeks of diazepam treatment. The amount of $\beta2$-subunit mRNA was increased in hippocampus after 2 weeks of treatment, and at withdrawal day 9 a significant rebound decrease in the levels of both the $\gamma2$- and $\beta2$-subunit mRNAs was found.

Thus, the effects of diazepam treatment on $GABA_A$ receptor steady-state mRNA levels are complex. The levels of different mRNA species may change in opposite directions within the same brain region at the same time, or, the level of a single mRNA species may simultaneously change in opposite directions in different brain regions (figure 2.4). Furthermore, in different brain regions, the level of a particular transcript may change in the same direction, but on clearly different timescales (figure 2.5).

DISCUSSION

The effect of benzodiazepines on $\mathrm{GABA_A}$ receptor steady-state mRNA levels has been a subject of interest for several years. The modified expression of these genes in the presence of an allosteric modulator suggests a homeostatic response of the neuron to pharmacological stimulation and, as such, may be a mechanism by which the brain becomes tolerant to the drug, protecting itself from an environmental insult.

Although this is the first comprehensive study in which the effects of diazepam on the expression of all of the α–, β– and γ-subunit isoforms known to exist in rat brain have been co-investigated, several groups have previously examined changes in the levels of specific $\mathrm{GABA_A}$ receptor subunit mRNAs after chronic diazepam exposure. Initially, Heninger *et al.* (1990) reported a significant decrease in α1-subunit mRNA levels in rat cortex after chronic diazepam exposure, but no changes in the level of this transcript in hippocampus or cerebellum. No changes were found in the level of β1-subunit mRNA in any of these three brain regions. In a subsequent publication, this group reported a significant decrease in the level of γ2-subunit mRNA in rat cortex, but not hippocampus or cerebellum, after equivalent diazepam treatment (Primus and Gallager, 1992). Wu *et al.* (1994) have reported significant decreases in α5-subunit mRNA levels in rat cortex and hippocampus following chronic diazepam treatment. A significant decrease in the level of γ2-subunit mRNA exclusively in cortex, and a significant decrease in the level of α1-subunit mRNA exclusively in hippocampus were also found. No significant changes in the levels of α1-, α2-, α3-, α4-, β2- or β3-subunit transcripts were observed. In the studies performed by the above groups crystalline diazepam was administered to rats for 21 days via implanted silastic capsules which provided continuous drug release, and $\mathrm{GABA_A}$ receptor steady-state mRNA levels were determined by northern blotting or, in the studies by Heninger *et al.* (1990) and Primus *et al.* (1992), by both northern blotting and solution hybridization. Impagnatiello *et al.* (1996) have reported a decrease in α1- and γ2- and an increase in α5- subunit mRNA levels in specific areas of the of the rat cortex, with no observed changes in the levels of α2-, α3-, α6-, β2- and γ1-subunit transcripts, following multiple daily diazepam injections. No changes in the level of any of these transcripts were observed in cerebellum, and the only change observed in hippocampus was a decrease in the level of α1-subunit mRNA. The technique this group used to measure $GABA_A$ receptor steady-state mRNA levels was RT-PCR.

The results of studies conducted by other groups, taken with the data presented in the current study, indicate much diversity in the reported effects of chronic diazepam treatment on the expression of $GABA_A$ receptor subunit genes. There are several possible explanations for this. Firstly, changes in mRNA levels have been shown to be time dependent (figure 2.5). Conflicting results between studies may, therefore, be due in part to different lengths of diazepam treatment before mRNA levels were measured. Secondly, the effects of diazepam on $GABA_A$ receptor gene expression may be sensitive to the specific level and timecourse of benzodiazepine binding site occupation. These parameters are not comparable between studies due to the fact that different diazepam doses and dosing methods (single daily injection versus multiple daily injection versus continuous release) have been used. Thirdly, considering the fact that Impagnatiello *et al.* (1996) report different effects of diazepam on $GABA_A$ receptor gene expression within different parts of a single brain region, the rat cortex, plus the fact that expression clearly varies between brain regions (table 2.2, figure 2.4), some of the variability in the reported effects of chronic diazepam treatment on $GABA_A$ receptor gene expression may result from investigators using different brain dissection methods or simply defining brain region boundaries differently. Finally, various techniques (northern hybridization, solution hybridization and quantitative PCR) with variable sensitivity and reproducibility have been used to measure mRNA levels in separate studies, which could contribute further to between-study inconsistencies. Of note, the present study and subsequent studies described in this thesis are internally consistent in terms of animal dosing, brain dissection, and the conditions of various assays.

Some important insights regarding the mechanism by which diazepam exposure changes GABA$_A$ receptor steady-state mRNA levels are provided by the present study. *In vitro*, diazepam does not interact with $\alpha_x\beta_2\gamma_2$ GABA$_A$ receptor constructs which contain the $\alpha4$ subunit. Yet, *in vivo*, the consequences of diazepam treatment include an increase in the steady state level of $\alpha4$-subunit mRNA (Wisden *et al.*, 1991). This finding suggests that the changes observed in GABA$_A$ receptor mRNA levels are not necessarily a direct consequence of diazepam on gene expression in individual cells, but rather could be the consequence of changes in GABAergic tone caused by interaction of the drug elsewhere in GABAergic pathways. Assuming conserved intracellular signaling pathways between cortical and hippocampal neurons, this interpretation is supported by the fact that there is a decrease in the steady state level of $\gamma2$-subunit mRNA in the cortex but an increase in the hippocampus .

Also of considerable interest is the observation that most of the diazepam-induced changes in GABA$_A$ receptor steady-state mRNA levels seen after one and two weeks of treatment are attenuated by the end of the four week treatment regime. Intuitively, one might expect a change in the steady-state level of a given mRNA species that occurs as a drug response to be equally or more robust upon extended drug exposure. There are at least two possible explanations for the transient nature of the changes presently observed, the first of which concerns the development of diazepam tolerance. If diazepam-induced changes in GABA$_A$ receptor gene expression are unrelated to the development of tolerance, yet dependent upon diazepam's modulatory effect at the receptor, then loss of the efficacy of this drug through the development of tolerance by some other mechanism would render it ineffective in sustaining changes in gene expression. Alternatively, changes in GABA$_A$ receptor subunit protein that are the result of changes in steady-state mRNA levels may persist in the presence of the drug, even if the mRNA levels themselves do not. This possibility will be further discussed in chapter four.

Chromosomal mapping studies have revealed that GABA_A receptor subunit genes tend to be organized as α/β/γ clusters. Specifically, the α1-, α6-, β2-, and γ2-subunit genes have been localized to human chromosome 5q32-5q33 (Buckle *et al.*, 1989; Wilcox *et al.*,1992; Hicks *et al.*, 1994; Russek and Farb, 1994), the α2-, α4-, β1- and γ1-subunit genes are clustered on human chromosome 4p13-4q11 (Buckle *et al.*, 1989; Kirkness *et al.*, 1991; Wilcox *et al.*, 1992; McLean *et al.*, 1995), and an α5-, β3- and γ3-subunit gene cluster exists on human chromosome 15q11-15q13 (Wagstaff *et al.*, 1991a; Knoll *et al.*, 1993). The α3-subunit gene exists alone on human chromosome Xq28 (Buckle *et al.*, 1989). Although no gene mapping studies have been done in the rat, there is evidence to suggest the presence of identical clusters in this species. GABA_A receptor subunit gene sequences and gene structures are highly conserved between vertebrates (Lasham *et al.*, 1991; Barnard *et al.*, 1993; Burt, 1994) and, where it has been determined, GABA_A receptor subunit gene clusters have been shown to be conserved in regions of the mouse genome that are syntenic with the human genome (Searle *et al.*, 1987; Wagstaff *et al.*, 1991b; Buckwalter *et al.*,1992; Nakatsu *et al.*, 1993; Danciger *et al.*, 1993). The data presented in this chapter suggest an association between the expression of different clusters of GABA_A receptor genes and diazepam treatment. This is illustrated in figure 2.6. Figure 2.6 (A) shows average GABA_A receptor subunit mRNA levels in cortex measured after 14 days of diazepam exposure, and grouped according to subunit gene cluster. At this time point mRNA changes were maximal in cortex. Figure 2.6 (B) illustrates diazepam's inverse effect in cortex and hippocampus on expression of the clustered α1-, β2- and γ2-subunit genes.

To determine whether these coordinate effects are due to synchronous transcriptional activation or, alternatively, due to the activation of selected genes in different neuronal populations within the brain, investigation using homogeneous cell systems will be required. There is, however, additional evidence which suggests the expression of clustered $GABA_A$ receptor genes may in some cases be co-regulated. This is most apparent for those genes that encode the α1, β2 and γ2 subunits. The transcripts of these genes are the most abundant in the adult rat brain and tend to co-localize (Wisden *et al.*, 1992). Further, levels of the α1- and γ2-subunit mRNA species in rat cortex have both been found to fall with chronic diazepam exposure and rise in response to chronic treatment with the benzodiazepine inverse agonist FG 7142 (Primus and Gallager, 1992). Three of the genes clustered on human chromosome 4, namely the α4-, β1- and γ1-subunit genes, demonstrate parallel expression during embryonic neurogenesis in the rat spinal cord (Ma *et al.*, 1993). These are the only transcripts expressed in the undifferentiated neuroepithileum, and, after birth, their abundance in spinal cord falls sharply. The α5-, β3- and γ3-subunit genes also show similar temporal patterns of expression through development (Laurie *et al.*, 1992). There are, however, patterns of $GABA_A$ receptor gene expression which are clearly not cluster dependent. Most notably the α6-subunit gene is expressed exclusively in the cerebellum of adult rats (Wisden *et al.*, 1992) in spite of the fact that the α6-subunit gene is clustered with the α1-, β2- and γ2-subunit genes. The mechanism of co-regulation of clustered genes is not understood. However, it is interesting to speculate that the expression of each cluster of $GABA_A$ receptor genes is regulated, at least in part, by a single control region in a fashion similar to that which has been described for other gene families. For example, the human globin gene locus is comprised of five genes which are expressed in a developmental- and tissue-specific manner. The expression of these genes, which occupy a 60 kb region of the short arm of chromosome 11, has been found to be influenced by a single locus control region located 50 kb upstream of the most 5' member (Grosveld *et al.*, 1987). Additional examples of gene loci which are regulated by locus control regions include the rat growth hormone gene locus (Aizawa *et al.*, 1995) and the mouse immunoglobin heavy-chain gene locus (Madisen and Groudine, 1994).

In summary, this study has shown that chronic diazepam exposure has the potential to modify the steady-state level of several GABA$_A$ receptor mRNAs in the rat brain in a time, subunit and brain region specific manner and, in addition, a degree of association between changes in the levels of mRNAs arising from a given gene cluster has been noted. In subsequent chapters, elements of the transcriptional basis and translational consequences of these changes will be examined, as will the question of whether or not these changes might be related to the clinical phenomena of benzodiazepine tolerance and dependence.

Transcript Specificity	Oligonucleotide Sequence	Nucleotides	Reference
α1	5'>GGGGTCACCCCTGGCTAAGTTAGGGGTATAGCTGGTTGCTGTAGG<3'	1166-1210	Khrestchatisky et al. (1989)
α2	5'>AGATTCGGGGCGTAGTTGGCAACGGCTACAGCA<3'	1445-1477	Khrestchatisky et al. (1991)
α3	5'>CTCAGCAGGACTGTCTTGCACATAAGTGGTCTTGGGGGAAGCAACACTG<3'	1533-1582	Malherbe et al. (1990)
α4	5'>CAAGTCGCCAGGCACAGGACGTGCAGGAGG<3'	57–86	Wisden et al. (1991)
α5	5'>CACAGCATTCCCAGTCCCGCCTGGAAGCTGCTCCTTTGGGA<3'	1485-1525	Malherbe et al. (1990)
α6	5'>CGTTGATGGTAAGATGGGCGTTCTACTGAGGACTTTGCTGGCCTCAGAAGATGGAACGAT<3'	1141-1200	Luddens et al. (1990)
β1	5'>ATGGCAACCATCACAGGAAAAGAGAGAAGCCCCAAACTCTCTCGA<3'	95-139	Ymer et al. (1989)
β2	5'>TCGTTCCAGGGCGTTGCGGCCAAAACTATGCCTAGGCAACC<3'	96-136	Ymer et al. (1989)
β3	5'>CTGAATTCCTGGTGTCACCAACGCTGCCTGCAACCTCATTCATTTCATTGTGAAC<3'	1190-1244	Ymer et al. (1989)
γ1	5'>GCAGTCTTCAAAGCAACAGAAAAAGGTAGCACAGTCTTTGCCCTCCAAGC<3'	1217-1266	Ymer et al. (1990)
γ2	5'>GTTCATTTGGATCGTTGCTGATCTGGGACGGAT<3'	1183-1215	Shivers et al. (1989)
γ3	5'>AGAGGGTGCTTAAGGCTTATTCGATCAGGAATCCATCTTGTTGAATCTGGATGT<3'	1170-1224	Herb et al. (1992)
β-actin	5'>CTGGTGGCGGGTGTGGACCGGGACGGAGGAGCTGCAA<3'	272-308	Nudel et al. (1983)

Table 2.1. Sequences and positions in the given references of the oligonucleotide probes used to quantify, by solution hybridization, GABA$_A$ receptor subunit steady-state mRNA levels.

Figure 2.1. Determination of the range within which there is a linear relationship between radioactivity and band intensity. A radiolabeled oligonucleotide (complimentary to the $GABA_A$ receptor α1-subunit mRNA) was prepared and serial dilutions separated by polyacrylamide gel electrophoresis. The resultant autoradiogram (BioMax X-ray film) was analyzed as described in the text to determine the integrated band optical densities. Panel A: Autoradiogram of gel-separated serial dilutions of a radiolabeled oligonucleotide. Lane 1, 0 dpm; lanes 2 to 9, 1177 to 1.5×10^5 dpm in 2-fold dilutions. Panel B: The integrated band intensities of the bands shown in (A) plotted against the amount of radioactivity loaded per lane. Panel C: As in (B) using only the data from the first seven lanes. The regression line has an R value of 0.997 ($P<0.05$). This figure has been reproduced from Bateson and Tanay (in press).

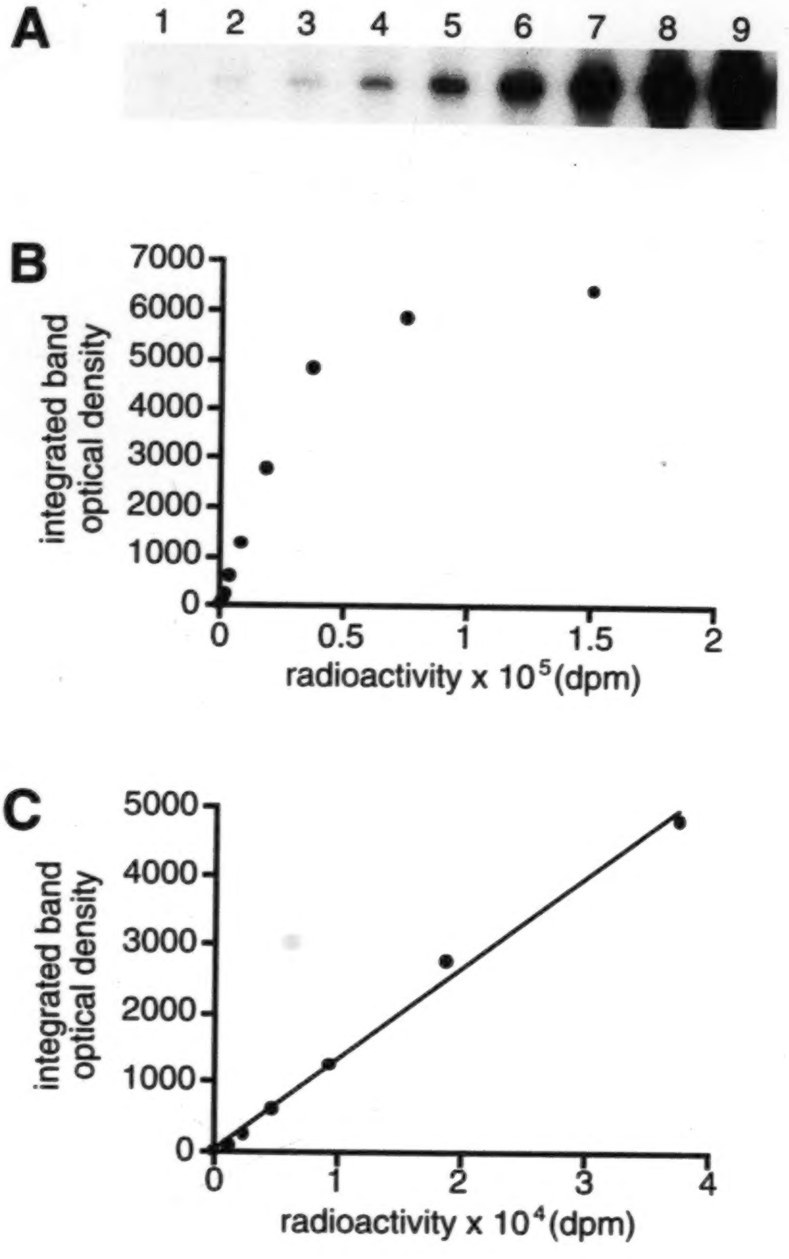
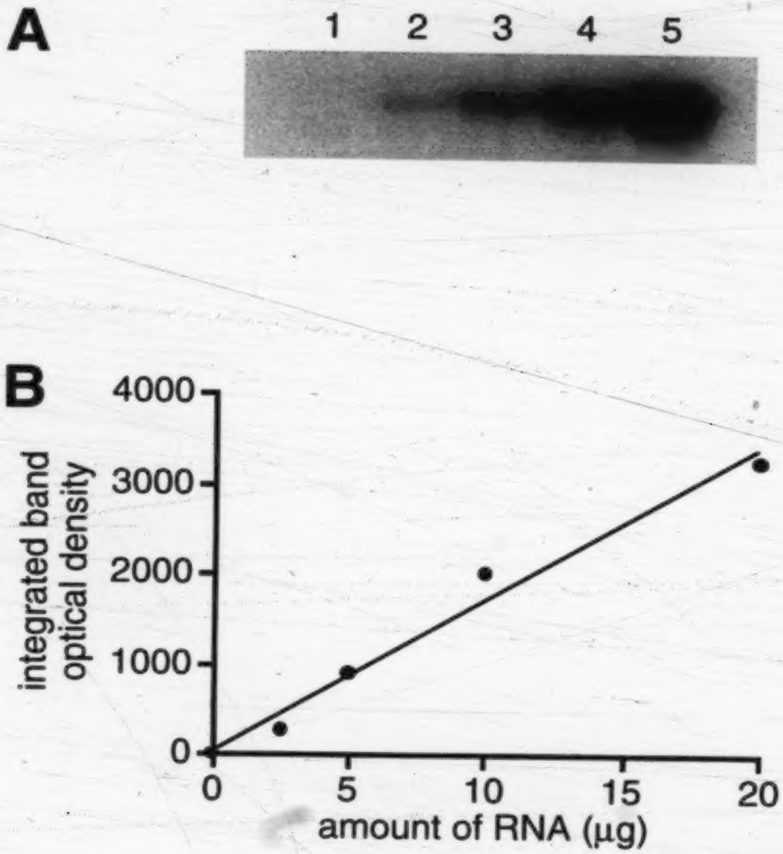

A
1
2
3
4
5
6
7
8
9
B
integrated band optical density
7000
6000
5000
4000
3000
2000
1000
0
0
0.5
1
1.5
2
radioactivity x 10^5 (dpm)
C
integrated band optical density
5000
4000
3000
2000
1000
0
0
1
2
3
4
radioactivity x 10^4 (dpm)

Figure 2.2. Determination of the range within which there is a linear relationship between mRNA level and band intensity. A standard solution hybridisation assay was performed using a constant amount of a radiolabeled β-actin-specific oligonucleotide and varying amounts of total RNA from rat cortex. The total RNA content in each assay tube was adjusted to 20 μg by the addition of an appropriate amount of yeast tRNA. Panel A: Autoradiogram showing that the amount of β-actin-specific oligonucleotide protected varied according the amount of RNA present. Total RNA from rat cortex: lane 1, 0 μg; lane 2, 2.5 μg; lane 3, 5 μg; lane 4, 10 μg; lane 5, 20 μg. Panel B: The integrated intensities of the bands shown in (A) plotted against the amount of rat cortical RNA per assay. The regression line has an R value of 0.990 ($P<0.05$). This figure has been reproduced from Bateson and Tanay (in press).


A
1
2
3
4
5
B
4000
3000
2000
1000
0
integrated band optical density
0
5
10
15
20
amount of RNA (µg)

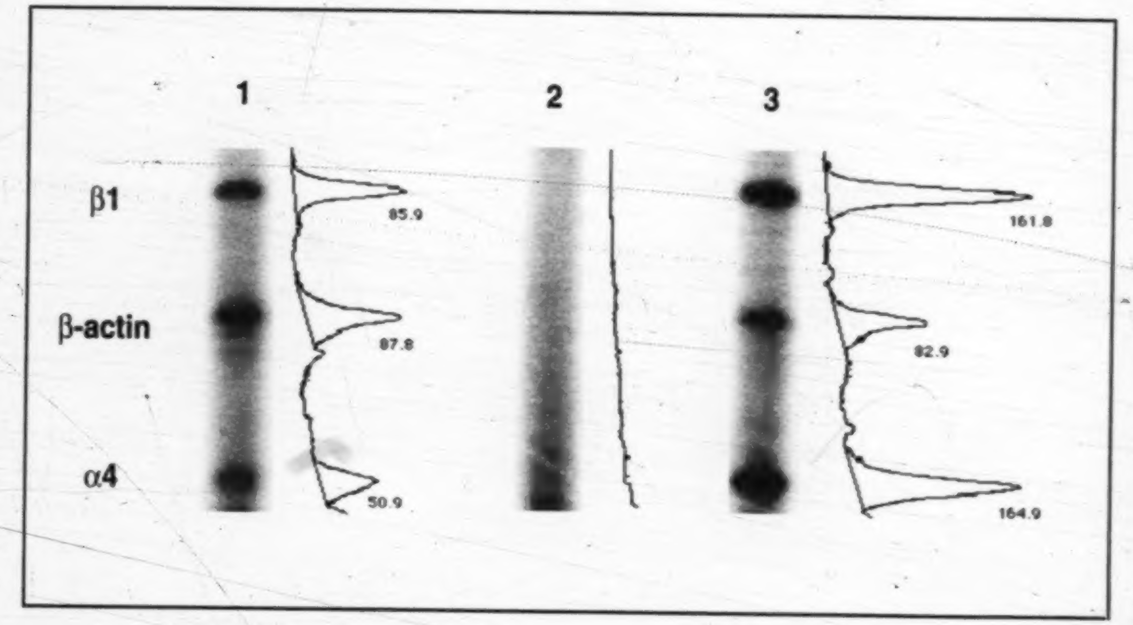


Figure 2.3. Selected lanes from an autoradiogram and their corresponding density plots illustrating results typical of an S1 nuclease protection experiment. Bands represent the amounts of GABA$_A$ receptor α4- and β1-subunit , and β-actin mRNA in samples of total RNA extracted from rat cortex. Integrated band intensities are in optical density units. Lane 1: hybridization using total RNA extracted from the cortex of an untreated control animal used in the hybridization reaction, lane 2: yeast tRNA was used in place of rat cortical RNA in the hybridization reaction, as a negative control, and lane 3: hybridization using total RNA extracted from the cortex of a rat treated for 14 days with 15 mg/Kg/day diazepam.

Table 2.2 The effect of chronic diazepam treatment and withdrawal on GABA$_A$ receptor subunit steady-state mRNA levels in rat cortex and hippocampus. The mean ratios of GABA$_A$ receptor subunit band intensities to β-actin band intensities are expressed as a percentage of untreated control values (+/- SEM). At each time point, different drug treatment groups (i.e. diazepam plus abecarnil or zolpidem; the effects of which are discussed in chapter five) were typically assayed together. Statistical comparisons between drug treatment and control groups were carried out using one way analysis of variance followed, if significance was achieved, by Newman Keuls test for multiple comparisons. (n=4-6, *$P<0.05$).

Cortex

subunit	7 days	14 days	28 days	28 days & withdrawal
α1	82.4 +/- 3.8	83.3 +/- 12.1	93.6 +/- 13.4	111.8 +/- 8.1
α2	100.9 +/- 8.4	120.1 +/- 11.5	101.2 +/- 15.2	80.7 +/- 5.6
α3	101.1 +/- 8.3	*136.0 +/- 1.0	138.9 +/- 20.5	71.4 +/- 4.9
α4	*181.7 +/- 19.2	*150.4 +/- 12.9	95.6 +/- 18.2	106.5 +/- 10.7
α5	136.8 +/- 10.3	*141.6 +/- 10.9	94.5 +/- 9.7	92.0 +/- 2.8
β1	*153.6 +/- 14.5	*131.9 +/- 7.9	90.0 +/- 11.8	89.1 +/- 9.7
β2	90.6 +/- 9.1	72.8 +/- 16.1	76.8 +/- 11.5	113.2 +/- 13.8
β3	96.6 +/- 25.0	106.2 +/- 2.8	95.6 +/- 11.9	99.5 +/- 2.8
γ1	134.0 +/- 18.8	100.7 +/- 21.9	111.4 +/- 15.5	88.5 +/- 9.4
γ2	93.1 +/- 9.5	*73.7 +/- 6.5	90.6 +/- 13.5	101.5 +/- 10.3
γ3	*161.3 +/- 27.8	*156.7 +/- 9.2	78.1 +/- 11.8	88.4 +/- 8.7

Hippocampus

subunit	7 days	14 days	28 days	28 days & withdrawal
α1	115.6 +/- 4.4	105.0 +/- 9.2	105.5 +/- 3.0	88.3 +/- 10.0
α2	94.8 +/- 5.6	121.1 +/- 8.2	118.1 +/- 8.3	75.2 +/- 17.1
α3	112.7 +/- 7.5	119.7 +/- 8.2	71.3 +/- 18.2	77.5 +/- 14.2
α4	130.4 +/- 30.4	165.8 +/- 15.2	*166.8 +/- 5.2	102.3 +/- 19.1
α5	108.8 +/- 10.2	120.5 +/- 9.1	108.6 +/- 2.4	85.1 +/- 2.6
β1	90.0 +/- 8.3	117.8 +/- 9.8	*211.5 +/- 7.1	68.3 +/- 17.8
β2	103.8 +/- 8.1	*150.5 +/- 3.4	102.0 +/- 10.2	*49.8 +/- 18.4
β3	85.9 +/- 8.8	98.5 +/- 8.1	101.4 +/- 1.4	119.3 +/- 3.4
γ2	*160.3 +/- 11.6	*156.3 +/- 7.9	145.9 +/- 16.9	*21.9 +/- 14.7

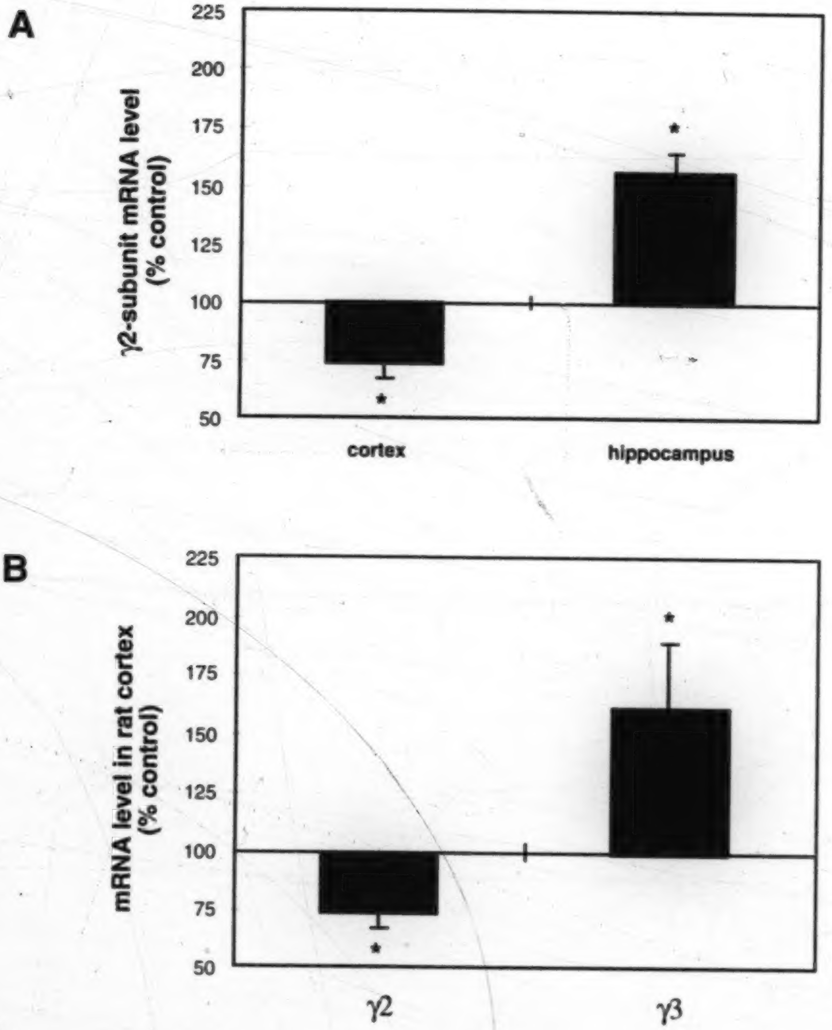


Figure 2.4. Examples of subunit- and brain region-specific changes in GABA_A receptor steady-state mRNA levels following chronic diazepam exposure. The mean ratios of GABA_A receptor subunit band intensities to β-actin band intensities are expressed as a percentage of untreated control values (+/- SEM). Statistical treatment was as described in table 2.2. (n=4-6, $^*P<0.05$)

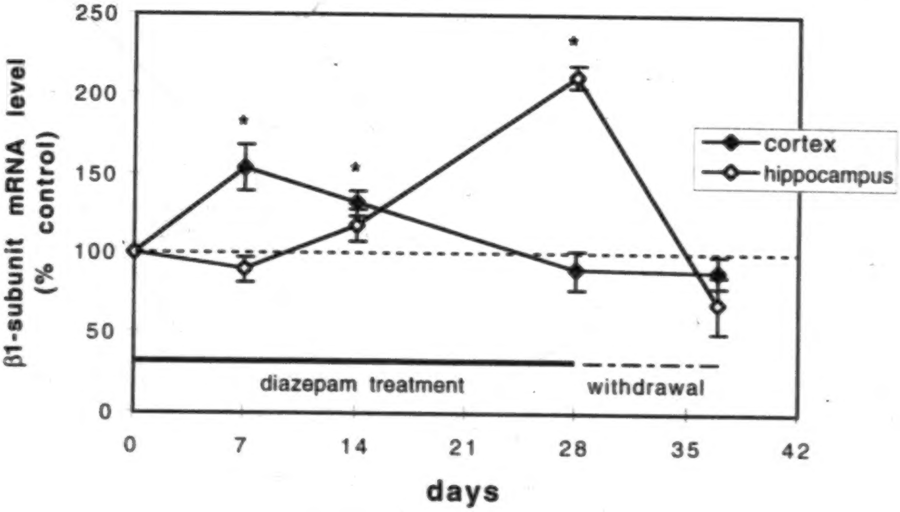
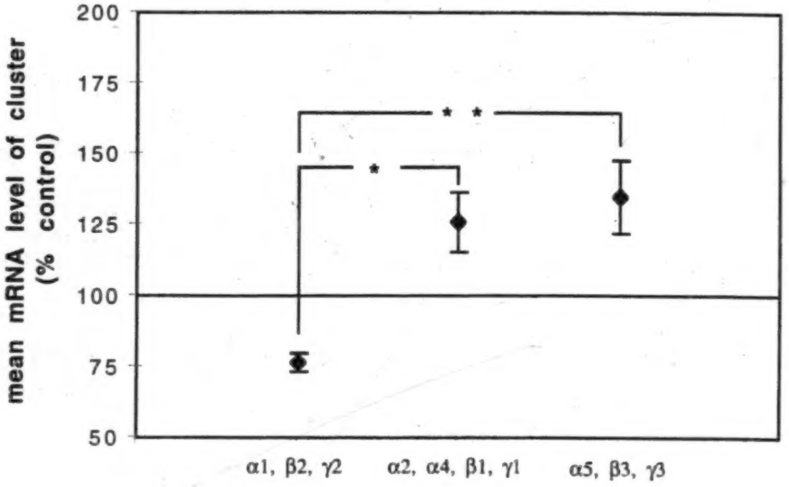


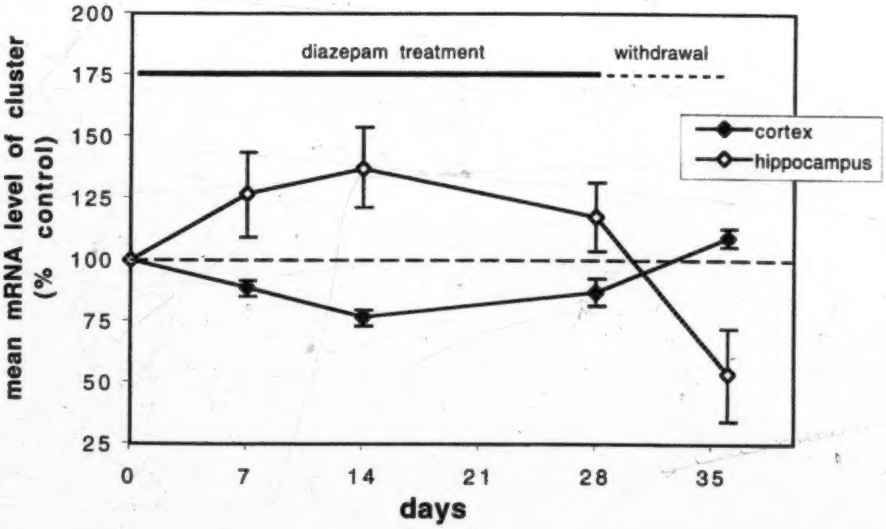
Figure 2.5. Timecourse of changes in $GABA_A$ receptor β1-subunit mRNA levels. The mean ratios of $GABA_A$ receptor subunit band intensities to β-actin band intensities are expressed as a percentage of untreated control values (+/- SEM). Statistical treatment was as described in table 2.2. (n=4-6, *$P<0.05$)

Figure 2.6. Association between the mean steady-state mRNA level of clustered $GABA_A$ receptor genes following chronic diazepam exposure. Panel A: cortical $GABA_A$ mRNA levels measured after 14 days of diazepam, as shown in table 2.2., have been regrouped according to human gene clusters and compared using Newman-Keuls test ($p<0.05$). Error bars represent the SEM for the values defining a cluster average. Mean changes in expression of the α5-, β3- and γ3-subunit clustered genes are not significantly different from mean changes in expression of the α2-, α4-, β1-, and γ1-subunit clustered genes but each of these means is significantly different ($p<0.01$, $p<0.05$ respectively) from the average change in expression of the α1-, β2- and γ2-subunit clustered genes. Panel B: the mean change in expression of the clustered α1-, β2- and γ2-subunit genes in cortex and hippocampus in response to chronic treatment, and withdrawal from chronic treatment, with diazepam.

A


mean mRNA level of cluster
(% control)
200
175
150
125
100
75
50
* *
*
α1, β2, γ2
α2, α4, β1, γ1
α5, β3, γ3

B


mean mRNA level of cluster
(% control)
200
175
150
125
100
75
50
25
diazepam treatment
withdrawal
cortex
hippocampus
0
7
14
21
28
35
days

Chapter 3

The effect of diazepam on transcription of the $GABA_A$ receptor γ2-subunit gene.

Data presented in this chapter have been previously published. Holt, R.A., I.L. Martin and A.N. Bateson, 1997, Mol. Brain Res. 48, 164.

INTRODUCTION

The mechanism by which $GABA_A$ receptor expression is regulated is currently unknown. This chapter describes experiments which have addressed the specific question of whether or not the transcription of a $GABA_A$ receptor gene can be modified in rat brain by exposure of the animal to diazepam. These experiments were conducted using the nuclear run-off transcription assay, the utility of which lies in its specificity for measuring the rate of transcription of a gene of interest. In comparison, analysis of steady-state mRNA levels cannot distinguish between transcriptional and post-transcriptional regulation.

The $GABA_A$ receptor γ2-subunit gene has been chosen for three reasons. Firstly, a change in the steady-state level of this transcript in response to chronic diazepam treatment has been observed in the studies described in chapter two, as well as in studies conducted by several other groups (Impagnatiello *et al.*, 1996; Primus and Gallager, 1992; Wu *et al.*, 1994). Secondly, γ2-subunit mRNA is an abundant transcript (Wisden *et al.*, 1992) which is essential for its detection and quantification using this technique. Finally, the γ2 subunit is of critical importance to the pharmacology of the benzodiazepine site (Pritchett *et al.*, 1989a) and it is therefore reasonable to expect that this particular subunit may play a role in the cellular response to diazepam exposure.

As shown in chapter two, 14 days of diazepam exposure changes the steady-state level of $GABA_A$ receptor γ2-subunit mRNA bi-directionally in rat cortex [73.3% +/- 6.6%, 4, (mean +/- SEM, n) of untreated control values, $P<0.05$] and hippocampus (156.3% +/- 7.9%, 4, $P<0.05$). A sufficient number of nuclei for a run-off experiment could not be obtained from rat hippocampus; therefore, to address the question of whether diazepam can inversely affect $GABA_A$ receptor gene transcription in different brain regions, nuclear run-off assays were conducted using nuclei derived from rat cortex or cerebellum. In cerebellum, the mean steady-state level of γ2-subunit mRNA following 14 days of diazepam exposure was 157.8% +/- 13.0% , 6, $P<0.05$ of untreated control values.

MATERIALS AND METHODS

Drug treatment

Adult, male, Sprague-Dawley rats were injected subcutaneously once daily for 14 days with 15 mg/kg diazepam in 1 ml of sesame oil vehicle, as described in chapter two, and killed 12 hours after their final dose. To determine whether diazepam exposure acutely affected transcription of the γ2-subunit gene, additional groups of rats were injected once with diazepam (15 mg/kg in 1 ml of sesame oil) and killed either 12 or 24 hours later.

Isolation of nuclei

At specific times after their last dose animals were sacrificed by decapitation and cortex and cerebellum were removed. Nuclei were isolated immediately following dissection by Dounce homogenization (10 to 20 strokes) of cortical or cerebellar tissue in buffer containing 0.32 M sucrose, 10 mM Tris-HCl (pH 8), 3 mM $CaCl_2$, 2 mM $MgAc_2$, 0.1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM spermidine, 1 mM dithiothreitol and 0.1% (v/v) Triton X-100. Nuclei were purified by centrifugation for 50 min at 16000 x g, 4°C, through a cushion of 3 M sucrose which contained the same protease inhibitors as the homogenization buffer. Purified nuclei were resuspended in 200 µl of storage buffer containing 40% (v/v) glycerol, 10 mM Tris-HCl (pH 8), 5 mM $MgCl_2$ (and protease inhibitors as above) and frozen at -80°C until required. Nuclei concentration was determined by counting the number of nuclei present in a 50:1 dilution of each sample using a hemacytometer, and nuclei purity and integrity were confirmed using phase-contrast microscopy.

Synthesis of nascent radiolabeled RNA

Approximately 1×10^7 nuclei from each animal were incubated with 25 µl of 10 mCi/ml, 3000 Ci/mmol α-^{32}P-UTP (Amersham) in 210 µl reaction buffer (5 mM Tris-HCl, pH 8, 2.5 mM $MgCl_2$, 0.15 M KCl and 250 µM ATP, CTP, and GTP), digested for 10 min at 30°C with 375 U of RNase-free DNaseI (Worthington) followed by digestion for 45 min at 50°C with 100 µg of proteinase K. Labeled transcripts were extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), precipitated for 30 min at -20°C in an equal volume of isopropanol and 2 M NH_4OAc, and centrifuged for 30 min at 12000 x g, 4°C). Pellets were resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) and this volume was applied to a 1 ml sephadex G-50 spin-column and centrifuged for 4 min at 1300 x g to remove unincorporated α-^{32}P-UTP.

Preparation of DNA probes

The probe used to detect nascent $GABA_A$ receptor γ2-subunit RNA was a 1.5 kb rat $GABA_A$ receptor γ2-subunit cDNA sequence generated by PCR in the following manner. 50 µl of PCR mix was prepared, containing 2 ng of template DNA (pSP72 plasmid containing a full length rat γ2-subunit cDNA insert) 50 pmol each of the forward (5'>TTTAGGTGACACTATAG<3') and reverse (5'>TAATACGACTCACTATA<3') primers, complimentary to regions of pSP72 polylinker sequence, 200 µM each of dATP, dTTP, dCTP and dGTP, 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4) and 0.4

units of TAQ polymerase (Gibco-BRL). 50 µl of sterile paraffin was added to prevent sample evaporation during amplification. Following amplification (95°C for 1 min, 50°C for 1 min, 72°C for 2 min for 25 cycles, then 5 min at 72°C) on a thermocycler (Techne; model PHC-3), samples were column purified using affinity chromatography (Quiagen; QIAquick PCR purification kit) to remove salt, enzyme and residual primers. The concentration of DNA in the final product was determined by measuring the absorbence of a 1:100 dilution at 260 nm, given that 1 A_{260} unit is equivalent to 40 µg/ml of double stranded DNA. As each PCR reaction yielded approximately 0.5 µg of purified DNA, it was necessary to perform approximately 40 parallel reactions in order to obtain sufficient probe for a nuclear run-off experiment. The purity of PCR generated probe was verified by running an aliquot of the pooled reaction product on a 1% agarose gel and observing a single prominent 1.5 kb band, and the identities of PCR generated probes were verified using restriction enzyme digestion.

The probe used to detect nascent γ-actin RNA, used as an internal standard in these experiments, was a 0.7 kb rat γ-actin sequence generated by PCR from a cDNA insert in M13 plasmid using the same conditions as above, with forward (5'> AACAGCTATGACCATG<3') and reverse (5'>TGACCGGCAGCAAAATG>3') primers being complimentary to regions of the M13 mp19 polylinker sequence. Non-homologous salmon sperm DNA was used as a negative control.

Immobilization of probes on nylon membranes

Nylon membranes (Amersham N+) were pre-wet in 10 x SSC (1.5 M sodium chloride, 0.15 M sodium citrate) and placed in a commercial blotting apparatus (Biorad; model BIODOT-SF). DNA probes in 10 x SSC were denatured at 95°C for 5 min and placed immediately on ice. Denatured probes were applied to the wells of the blotting apparatus (2 μg of probe in a 200 μl volume, per well) in replicates of twelve such that twelve individual membranes, each blotted with a γ2-subunit probe, a γ-actin probe, and salmon sperm DNA were prepared simultaneously. After a 10 min room temperature incubation, the liquid was removed from the wells via suction through the membrane. Immediately following probe application membranes were placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min, then transferred to neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) for 1 min, air dried, and uv-crosslinked using a Fisher Scientific UV Crosslinker, model FB-UVXL-1000, set on "optimal crosslink".

Hybridization of radiolabeled RNA to membrane-immobilized probes

Each sample of α-^{32}P-UTP labeled RNA was incubated in hybridization buffer containing 5 x SSPE (0.75 M sodium chloride, 0.5 M sodium dihydrogen phosphate, 5 mM EDTA), 0.5% (w/v) sodium dodecyl sulphate, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) Ficoll-400, 0.1% polyvinylpyrrolidone and 0.02 mg/ml sonicated salmon sperm DNA, for 48 hours at 65°C with a membrane containing immobilized DNA probes. After hybridization the membranes were washed under low stringency conditions (two 10 minute washes in 2 x SSPE, 0.1% (w/v) SDS at 25°C and two 15 minute washes in 1 x SSPE, 0.1% (w/v) SDS at 25°C), followed by two high stringency washes (15 minutes each in 0.1x SSPE, 0.1% SDS at 65° C). After incubation for 30 minutes at room temperature in 2x SSPE containing 2 μg/ml RNase A, membranes were subjected to a final 10 min wash in 2 x SSPE, 0.1% (w/v) SDS at 25°C.

Detection and quantification of nascent γ2-subunit RNA

Air-dried membranes were opposed to a phospho-imaging plate and, following typically one week of exposure, band intensities were measured using a Bio-imaging analyzer (Fujix) running BAS 1000 MACBAS software. From each measurement the background of the appropriate membrane was subtracted and, for each sample, the intensity of the $GABA_A$ receptor γ2-subunit band was normalized to the intensity of the band produced by the γ-actin internal standard. Normalized values from the diazepam treatment group were compared with corresponding values from the control group.

RESULTS

In cortex, a significant decrease in the production of nascent γ2-subunit RNA in response to 14 days of diazepam treatment was observed. The magnitude of this decrease was to 35.3+/-3.4% (mean+/-SEM, n=5, $p<0.05$) of untreated control values (figure 3.1). The experiment was repeated, comparing diazepam-treated rats with vehicle-treated control animals, with very similar results (a decrease to 32.1+/-9.9%, n=5, $p<0.001$). No significant difference was observed between vehicle treated and untreated animals. In cerebellum, 14 days of diazepam treatment had an inverse effect, causing a significant increase in the production of nascent γ2-subunit RNA to 141.6 +/- 2.1% (n=4, $p<0.001$) of untreated control values (figure 3.1). No significant changes in nascent γ2-subunit RNA levels were observed 12 hours or 24 hours after a single dose of diazepam, in cortex or cerebellum (figure 3.2). Figure 3.3 shows the results of a typical nuclear run-off experiment. Note that the non-specific signal was indistinguishable from membrane background.

DISCUSSION

This is the first demonstration of a change in the transcription rate of a $GABA_A$ receptor gene in response to treatment with a $GABA_A$ receptor allosteric modulator. Our findings are consistent with the results of *in vitro* work undertaken by Kang *et al.* (1994). This group isolated a portion of the 5' end of the human $GABA_A$ receptor $\alpha1$-subunit gene which conferred benzodiazepine sensitive promoter activity in transiently transfected neurons. The potential for modification of the transcription of $GABA_A$ receptor subunit genes has been previously demonstrated by Harris *et al.* (1995) who showed that NMDA and potassium depolarization of primary cultures of cerebellar granule cells increases the transcription of genes encoding the $GABA_A$ receptor $\alpha1$ and $\alpha5$ subunits. Interestingly, subsequent work by this group has indicated that exposure of cultured cerebellar granule cells to NMDA also decreases the half-life of the $\alpha1$-subunit mRNA species (Ikonomovic *et al.*, 1996), suggesting that changes in transcription rate and mRNA stability can occur simultaneously. In other studies, however, in which cultured neurons were exposed to GABA, the steady-state level of $\alpha1$-subunit mRNA decreased but the half-life of this transcript did not change (Lyons and Farb, 1995).

The present study shows that the cellular response to chronic benzodiazepine stimulation includes modification of the rate of transcription of a $GABA_A$ receptor subunit gene. It is interesting that significant changes in transcription were not observed after a single diazepam dose, perhaps suggesting that the changes seen with chronic exposure are due to the cumulative effects of multiple doses. Alternatively, the mechanism by which diazepam signals transcriptional changes in the nucleus may be slowly propagated, such that there is a lag between initial exposure to the drug and transcriptional effects. Diazepam-induced changes in transcription of the $\gamma2$-subunit gene concur with changes in $\gamma2$-subunit steady-state mRNA levels in cortex and cerebellum measured after an equivalent diazepam treatment schedule. While these results do not preclude the possibility that the $\gamma2$-subunit transcript is subjected to post transcriptional regulation (i.e. modification of mRNA half-life), it is clear that transcriptional control is important in mediating the response of this gene to diazepam.

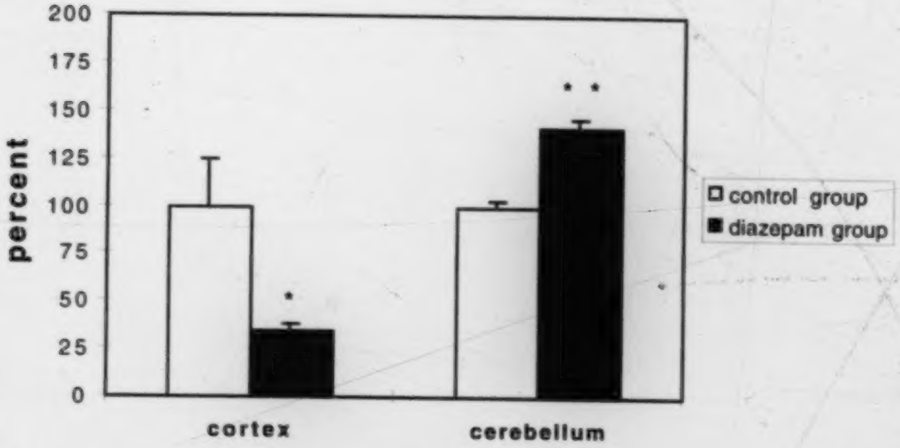


Figure 3.1. The effect of 14 days of diazepam exposure on transcription of the $GABA_A$ receptor γ2-subunit gene in rat cortex and cerebellum. Data are expressed as a percentage of the mean control value (+/- SEM). Normalized values from the diazepam treatment group were compared with corresponding values from the control group using Student's two-tailed unpaired t-test (*P<0.05; cortex, n=5; cerebellum, n=4, P<0.001).

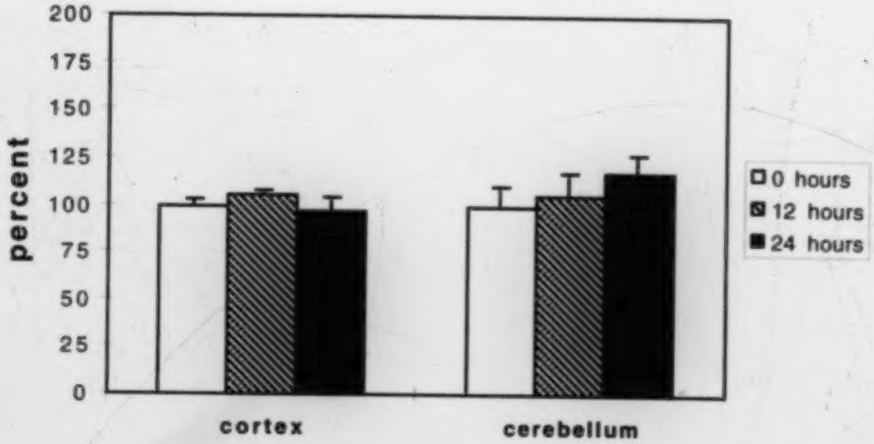


Figure 3.2. Transcription of the $GABA_A$ receptor γ2-subunit gene in rat cortex and cerebellum measured 12 or 24 hours after a single dose of diazepam. Data is expressed as a percentage of the mean control value (+/- SEM). Separately, in cortex and cerebellum, normalized values from the diazepam treatment groups were compared with the corresponding value from the control group using one way analysis of variance. There were no significant effects at P=0.05 (cortex, n=5, 3, 6 for the 0, 12 and 24 h time points, respectively; cerebellum, n=8, 6, 8 for the 0, 12 and 24 h time points, respectively).

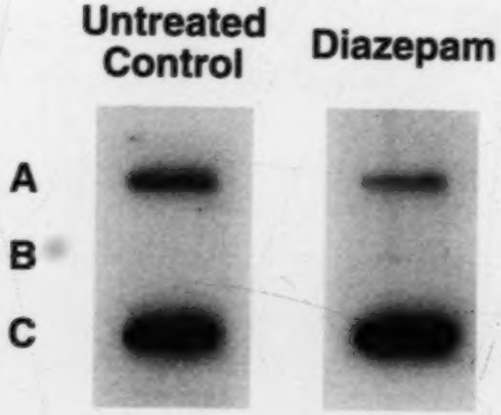


Figure 3.3. Representative phospho-image illustrating the results of a typical nuclear run-off experiment. These data show the effect, in cortex, of 14 days of diazepam exposure. Band intensities correspond to the radioactivity of nascent transcripts that hybridize to A) rat GABA$_A$ receptor γ2-subunit cDNA; B) sonicated salmon sperm DNA; and C) rat γ-actin cDNA.

CHAPTER 4

The effect of diazepam on benzodiazepine-site binding characteristics

PART 1: The effect of chronic diazepam on benzodiazepine recognition properties

INTRODUCTION

The results of the previous two chapters have been discussed with the presumption that diazepam-induced changes in $GABA_A$ receptor gene transcription and steady-state mRNA levels result in equivalent changes in receptor protein. Generally, considering the following evidence, this a reasonable assumption to make. The relative abundance and topographical localization of mRNA species encoding thirteen different $GABA_A$ receptor subunits has been determined in the adult rat brain using *in situ* hybridization (Wisden *et al.*, 1992). Likewise, immunological methods have been used to document the distribution of α1-, α2- and α3-subunit (Zimprich *et al.*, 1991; Fritschy and Mohler, 1995), α5-subunit (Thompson *et al.*, 1992; Fritschy and Mohler, 1995), α6-subunit (Thompson *et al.*, 1992), β2-subunit (Machu *et al.*, 1993), γ2-subunit (Fritschy and Mohler, 1995) and δ-subunit (Benke *et al.*, 1991) immunoreactivity. Taken together, these *in situ* hybridization and immunological studies suggest that in the adult rat brain $GABA_A$ receptor polypeptide expression generally parallels that of the corresponding mRNA. In addition, the distribution of BZI and BZII binding sites throughout the adult rat brain are largely predictable from regional differences in α-subunit mRNA levels (Young *et al.*, 1981). For example in the cerebellum (the brain region with the highest density of BZI binding sites) the concentration of mRNA for the α1 subunit, which confers BZI type pharmacology, is highest.

During ontogeny, there are marked changes in the expression of various $GABA_A$ receptor subunits. Developmental changes in the levels of 13 different subunit mRNAs (Laurie *et al.*, 1992; Gambarana *et al.*, 1991) and a limited set of subunit proteins (Fritschy *et al*; 1994; Nadler *et al.*, 1994), as well as variations in receptor binding properties (Lippa *et al.*, 1981), have been characterized. Generally, the levels of $GABA_A$ receptor subunit polypeptides increase in close parallel with their mRNAs during ontogeny, and the prominent levels at birth of α2-subunit mRNA and protein, plus the postnatal increase in

$\alpha 1$-subunit gene expression, correspond with changes in the relative abundance of BZII and BZI binding sites (Lippa *et al.*, 1981). Notably, however, $\beta 2/\beta 3$- and $\gamma 2$-subunit immunoreactivity in the cortex increases during development without any significant change in mRNA and, in the cerebellum, $\gamma 2$-subunit mRNA decreases significantly two weeks after birth while $\gamma 2$-subunit protein remains elevated (Gambarana *et al.*, 1991; Nadler *et al.*, 1994). These unpredicted results suggest that in addition to changes in mRNA levels, changes the efficiency of subunit translation, assembly or transport may affect $GABA_A$ receptor expression during ontogeny.

A final piece of information regarding the relationship between $GABA_A$ receptor subunit mRNA and protein levels is provided by a study in which primary cultures of cerebellar granule cells were transfected with $\alpha 6$- and $\gamma 2$-subunit antisense oligonucleotides (Zhu *et al.*, 1996). The levels of the $\alpha 6$- and $\gamma 2$-subunit proteins were reduced within 48 hours of oligonucleotide exposure, as evidence by decreased immunoreactivity and by changes in the binding characteristics of flunitrazepam and furosamide (an $\alpha 6$-subunit specific ligand).

In the present study, the functional consequences of modified receptor subunit composition are assessed in terms of benzodiazepine-site ligand recognition properties, in order to provide some experimental evidence that the diazepam-induced changes in $GABA_A$ receptor subunit mRNA levels can result in changes in receptor protein levels. While the proteins themselves could be quantified directly using immunological techniques, subunit-specific antibodies are difficult to make due to the high degree of sequence identity between the different subunit isoform. Antibody preparations with the required subunit specificity are not commercially available.

Differences in the benzodiazepine binding properties of recombinant $GABA_A$ receptors have been discussed in detail in chapter one, and have been reviewed by Sieghart (1995). In the present study, the following observations are of particular importance. While a γ subunit is necessary for benzodiazepine recognition, flunitrazepam binds with approximately 100-fold lower affinity to receptors which contain the γ3 subunit than to receptors which contain the γ2 subunit. Ro15-4513, a benzodiazepine site inverse agonist, binds with high affinity to both γ2 and γ3 subunit-containing receptor constructs. In addition, whereas flunitrazepam has equally high affinity for α1, α2, α3 and α5 subunit-containing receptors and essentially no affinity for α4 and α6 subunit-containing receptors, Ro15-4513 does not distinguish between receptors comprised of different α subunits. In the present study, comparisons have been made between the binding of flunitrazepam and Ro15-4513 to preparations of cortical rat brain membranes in order to obtain information regarding 1) the relative numbers of benzodiazepine sensitive and benzodiazepine insensitive $GABA_A$ receptors present and, by inference, 2) the abundance of the particular α and γ subunits that confer benzodiazepine sensitivity.

Zolpidem is a subtype specific benzodiazepine site ligand which differs from classical benzodiazepines by way of its approximately 150-fold lower affinity for α2- and α3 subunit-containing recombinant receptors. While zolpidem shows a complete lack of affinity for α5 subunit-containing receptors in recombinant systems, native $GABA_A$ receptors immunopurified from rat brain extracts using α5-subunit antibody show regionally variable zolpidem affinity; in hippocampus, α5 subunit-containing receptors are relatively insensitive to zolpidem, but in cortex, α5 subunit-containing receptors have affinity for zolpidem comparable to that of recombinant α2 and α3 subunit-containing receptors (Mertens et al., 1993). Perhaps these cortical receptors contain the α5 subunit in association with another α subunit. In the present study, experiments involving zolpidem displacement of ^{3}H-flunitrazepam from cortical rat brain membranes have been performed in order to estimate the relative numbers of type BZI (associated with α1 subunit-containing receptors) and type BZII (associated with α2, α3, and α5 subunit-containing receptors) binding sites. Unfortunately, the presence of zolpidem-sensitive α5 subunit-containing receptors in rat cortex precludes the specific measurement of BZIII binding sites (binding specifically to α5 subunit-containing receptors) using zolpidem displacement assays.

MATERIALS AND METHODS

Drug treatment

Animals (Male Sprague Dawley rats, initially 175 g to 200 g) were treated as described in chapter two, with single daily injections of 15 mg/kg diazepam in 1 ml of sesame oil vehicle for 14 or 28 days. To maintain consistency with the previously discussed gene expression studies, control rats were not injected. Following sacrifice, the cortex was removed from individual rats, immediately frozen in liquid nitrogen, then stored at -80°C.

Preparation of rat brain membranes

Frozen tissue was added to 20 volumes (v/w) of ice cold protease-inhibitor buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 µg/ml bacitracin, 50 µg/ml soya bean trypsin inhibitor and 100 µM phenylmethylsulfonyl fluoride), thawed on ice, then homogenized for 10 s using an Ultra Turrax homogenizer set on medium. Following homogenization, samples were incubated on ice for 10 min and then centrifuged for 20 min at 20000 x g, 4°C. Supernatants were discarded and each pellet was resuspended in 50 volumes (v/w) of 50 mM Tris-HCl buffer (pH 7.4) by brief homogenization (5 s, using the Ultra Turrax homogenizer set on medium), then incubated on ice, centrifuged and resuspended in Tris-HCl buffer as above. This process was repeated an additional three times to remove any residual diazepam or GABA. After the final centrifugation step, pellets were resuspended in 20 volumes of 50 mM Tris-HCl buffer (pH 7.4) and the protein concentration of each sample was determined (described below). Samples were frozen at -80°C until required.

Estimation of protein concentration in membrane preparations

The protein content of each membrane preparation was determined using a bicinchoninic colorimetric assay, according to the manufacturers instructions. Duplicate, 10 µl aliquots of each membrane sample were added to 1 ml of working reagent (containing 0.08% (w/v) copper sulfate) and incubated for 30 min at 37°C to allow color development. The absorbence of each sample was measured at 562 nm, and protein concentration was estimated by comparison with a standard curve constructed using known concentrations of bovine serum albumin.

Single point ^{3}H-flunitrazepam and ^{3}H-Ro15-4513 binding assays

Immediately prior to the binding assay membrane preparations were thawed and the tissue was dispersed by pulse homogenization. An aliquot of each membrane preparation containing 100 µg of protein was taken and the volume increased to 900 µl with 50 mM Tris-HCl (pH 7.4). For the flunitrazepam binding studies, ^{3}H-flunitrazepam (Dupont-NEN, specific activity 83.4 Ci/mmol) was added to a final concentration of 2 nM and a final volume of 1 ml. Non-specific binding was defined by incubating parallel reactions in the presence of 3 µM clonazepam. For the Ro15-4513 binding studies, ^{3}H-Ro15-4513 (Dupont-NEN, specific activity 83.4 Ci/mmol) was added to a final concentration of 10 nM and a final volume of 1 ml, and non-specific binding was defined by parallel reactions which included 10 µM unlabelled Ro15-4513. Samples were vortexed, incubated in ice-water for 2 h and then, using a Brandel M-24R cell harvester, rapidly filtered through glass fiber filter paper (Whatmann GF/B) and rinsed twice with 4 ml of ice cold 50 mM Tris-HCl (pH 7.4). The radioactivity retained on the filters was measured by liquid scintillation counting.

Single point zolpidem displacement binding assays

Single point ^{3}H-flunitrazepam binding experiments were performed as described above, but in the presence or absence of 100 nM zolpidem. At this concentration zolpidem will displace ^{3}H-flunitrazepam from α1 subunit-containing receptors and leave α2, α3 and α5 subunit-containing receptors labeled. Thus, specific ^{3}H-flunitrazepam binding in the presence of 100 nM zolpidem will give the number of BZII sites, and subtracting this value from the measured total specific ^{3}H-flunitrazepam binding gives the number of BZI sites. As above, non-specific binding was defined in the presence of 3 μM clonazepam.

RESULTS

The results of the single point flunitrazepam and Ro15-4513 binding experiments are illustrated in figures 4.1 and 4.2. No differences in the total binding of ligand were seen between rat brain membranes prepared from animals treated for 14 days with diazepam compared and those prepared from control animals (figure 4.1). In membrane preparations from the control group the ratio of the number of flunitrazepam to Ro15-4513 binding sites was 0.8 +/- 0.02 (mean+/- SEM, n=6) and no difference was observed between this value and the corresponding ratio in the drug treatment group (figure 4.2). After 28 days of diazepam exposure, total flunitrazepam binding was significantly decreased compared to control, whereas total Ro15-4513 binding was unchanged (figure

4.1). At this time point, the ratio of flunitrazepam to Ro15-4513 binding was also significantly reduced (figure 4.2). The results of the zolpidem displacement experiments are illustrated in figure 4.3. In comparison to control values, the ratio of specific BZII binding to specific BZI binding was unchanged after 14 days of diazepam treatment, but significantly increased after 28 days of exposure. Of note, exposure of animals to vehicle alone did not significantly effect total flunitrazepam binding or the ratio of BZII to BZI binding.

DISCUSSION

The data presented in part 1 of this chapter indicate that chronic diazepam exposure modifies the proportion of different GABA$_A$ receptor subtypes in rat cortex without, at the dose and time points examined, changing the total number of GABA$_A$ receptors present. These results are largely predictable from the diazepam-induced changes in GABA$_A$ receptor steady-state mRNA levels, discussed in chapter two. The decrease in total ^{3}H-flunitrazepam binding in cortex[3] shown in figure 4.1 is consistent with a reduction in the proportion of γ2 subunit-containing receptors present, as predicted from the effect of diazepam on the γ2-subunit gene transcription rate and steady-state mRNA level in this brain region (see figures 2.4 and 3.1). Figure 4.2 shows that flunitrazepam will label approximately 80% of the GABA$_A$ receptors labeled by Ro15-4513. Based upon what is known about the binding properties of recombinant receptors *in vitro*, the difference in total binding between these two ligands is likely due to the ability of Ro15-4513 to bind α4 subunit-containing receptors (the α6 subunit is absent in cortex) and γ3 subunit-containing receptors with high affinity. The fact that flunitrazepam binding decreases in rat cortex in response to chronic diazepam treatment, whereas Ro15-4513 binding does not, indicates that in addition to a reduced proportion of $\gamma 2$ subunit-containing receptors, there are likely increased proportions of $\gamma 3$ subunit-containing and/or $\alpha 4$ subunit-containing receptors. The steady-state mRNA levels for both of these subunits has been shown to increase in rat cortex in response to an identical diazepam dosing regimen (see table 2.2).

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[3] Previous studies have shown a decrease (Miller et al., 1988; Rosenberg and Chiu, 1981) or no change (Gallager et al., 1984) in ^{3}H-flunitrazepam binding to cortical membranes prepared from rats chronically exposed to benzodiazepines, and it is clear that the effect is dose dependent.

The zolpidem displacement experiments provide additional information regarding changes in the proportion of receptors containing different α-subunit isoforms. A significant increase in the ratio of BZII type binding (binding to receptors containing the $\alpha 2$-, $\alpha 3$- and $\alpha 5$-subunits) to BZI type binding (binding to $\alpha 1$ subunit-containing receptors) is seen in the cortex of diazepam treated rats, consistent with significant diazepam-induced increases in $\alpha 3$- and $\alpha 5$-subunit mRNA levels, as shown in table 2.2. Of note, a small decrease in the level of $\alpha 1$-subunit mRNA is also seen in rat cortex following chronic diazepam exposure (table 2.2), but this effect does not reach statistical significance at any time point.

Despite the low temporal acuity of the present experiments, the observed delay between changes in $GABA_A$ receptor steady-state mRNA levels in cortex and changes in receptor protein was unexpected. Differences in specific subunit mRNA levels are observed after one week of diazepam treatment, are generally maximal after two weeks of exposure, and return to baseline levels following the full 28 day dosing regime. However, no differences in receptor recognition properties are apparent until treatment has progressed for 28 days. Perhaps, because the effects of diazepam on message levels are small (i.e. generally less than 1.5-fold), changes in receptor protein are slowly realized. If, in contrast, diazepam were to radically alter $GABA_A$ receptor mRNA levels, then changes in receptor protein might be seen after a shorter exposure time. The time required for changes in mRNA abundance to alter protein levels has not been explicitly studied in the GABAergic system An alternate explanation for the discordant timecourse of diazepam-induced changes in GABA$_A$ receptor subunit mRNA and protein levels may be modification of the efficiency of mRNA translation. Episodes of discordance between changes in GABA$_A$ receptor mRNA and protein levels have been documented during development. For example, as noted in the introduction to this section, γ2-subunit immunoreactivity is maintained at a high level in neonatal rats even after the prenatal increase in γ2-subunit mRNA that induced the change has subsided (Gambarana *et al.*, 1991; Nadler *et al.*, 1994). Perhaps in this case, and/or in the case of diazepam induced changes in GABA$_A$ receptor expression, the efficiency of translation of GABA$_A$ receptor subunit polypeptide from mRNA is altered, either as a downstream effect of mRNA induction/repression *per se*, or via a mechanism unrelated to steady-state mRNA level. Translational regulation has been intensively studied in a number of model systems, and the importance of both *cis*-acting (e.g. variations in mRNA poly(A) tail length) and *trans*-acting (e.g. recruitment of translation initiation and elongation factors) control mechanisms has been revealed (reviewed by Hentze, 1995). For a number of metabolic enzymes, including ferritin (Gray and Hentze, 1994), ornithine decarboxylase (Ito *et al.*, 1990) and 15-lipoxygenase (Ostareck-Lederer *et al.*, 1994), translational control has been shown to be a principal determining factor in the rate of protein production, and to be sensitive to environmental cues.

In summary, the results presented in this section show that changes in benzodiazepine recognition properties occur in rat brain following chronic diazepam treatment. The proportions of receptors containing specific GABA$_A$ receptor subunit isoforms have been inferred from these data, and these proportions are largely predictable from the effects of diazepam on GABA$_A$ receptor steady-state mRNA levels.

PART 2: The effect of diazepam exposure on GABA enhancement of benzodiazepine binding

INTRODUCTION

The multiple distinct binding sites on the $GABA_A$ receptor for agents such as the benzodiazepines, barbiturates and certain neurosteroids are allosterically linked to each other, and to the GABA binding site (Sieghart, 1995). Perhaps the best studied $GABA_A$ receptor allosteric interaction is the so called "GABA shift" -- the enhancement, by GABA, of *in vitro* binding of radiolabeled benzodiazepine agonist to $GABA_A$ receptors present in homogenates of nervous tissue or cultured cells (Martin and Candy, 1978; Tallman *et al.*, 1978; Briley and Langer, 1978). Prolonged administration of benzodiazepine agonists *in vivo* (Gallager *et al.*, 1984; Tietz *et al.*, 1989), in primary neuronal culture (Friedman *et al.*, 1996; Hu and Ticku, 1994; Roca *et al.*, 1990) or in cell lines transfected with $GABA_A$ receptor cDNAs (Klein *et al.*, 1994; Klein *et al.*, 1995; Primus *et al.*, 1996; Wong *et al.*, 1994) reduces the strength of the allosteric interaction between the benzodiazepine and GABA binding sites, an effect that likely represents a homeostatic mechanism by which neurons adjust to the enhanced inhibitory tone conferred by the presence of the modulator. Chronic exposure to other classes of $GABA_A$ receptor modulators can also cause uncoupling effects, and these may be either homologous (affecting only the allosteric interactions of the binding site of the modulator being applied) or heterologous (affecting the allosteric interactions of multiple sites) in nature, depending on the particular modulator and experimental system used (Klein *et al.*, 1995; Friedman *et al.*, 1996).

To date, estimates of the timecourse of benzodiazepine-induced uncoupling of the benzodiazepine and GABA binding sites have been markedly different using different experimental paradigms. While uncoupling occurs quickly in cultured cells, with marked decreases in the GABA shift typically seen after less than 24 hours of benzodiazepine exposure (Klein *et al.*, 1994; Klein *et al.*, 1995; Primus *et al.*, 1996; Roca *et al.*, 1990; Wong *et al.*, 1994), experiments using brain homogenates prepared from animals chronically treated with benzodiazepines have been indicative of a chronic uncoupling effect manifested over a number of weeks, not hours, of exposure (Gallager *et al.*, 1984; Hernandez *et al.*, 1989; Tietz *et al.*, 1989).

The cellular mechanism responsible for uncoupling has not been determined, though one can speculate that changes in the phosphorylation state of the receptor, receptor internalization, or modification of receptor subunit composition may be involved. An understanding of the dynamics of uncoupling is important when trying to distinguish between possible causes of this phenomenon. In this section of chapter four the acute and chronic effects of diazepam on allosteric coupling of the GABA and benzodiazepine binding sites in rat brain are investigated. To determine whether uncoupling can be rationalized in terms of altered GABA receptor gene expression, results are compared to the effects of diazepam on transcription of the $GABA_A$ receptor $\gamma 2$ -subunit gene and steady-state $\alpha 1$, $\beta 2$ and $\gamma 2$-subunit mRNA levels.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats were treated acutely with single injections of 15 mg/kg diazepam in 1 ml of sesame oil vehicle and killed 1, 4, 12 or 24 h later. Chronically treated animals received 14 single daily injections of 15 mg/kg diazepam in 1 ml of sesame oil vehicle and were killed 24 hours after their final dose. Control rats in the chronic study were not injected so that results could be directly compared with the 24 hour time point of the acute study. Following sacrifice, the cortex and cerebellum were removed, immediately frozen in liquid nitrogen, then stored at -80°C. The preparation of membranes and estimation of protein content were performed as described in part 1 of this chapter. For the acutely treated animals single cortical or cerebellar membrane samples were prepared for each time point by pooling tissue from three different animals.

GABA shift binding assays

Binding assays measuring the potentiation of flunitrazepam binding by GABA were performed in the same manner as the single point flunitrazepam binding experiments described in part 1. Immediately prior to the binding assay membrane preparations were thawed and the tissue was dispersed by pulse homogenization. An aliquot of each membrane preparation containing 100 μg of protein was incubated with 2 nM ^{3}H-flunitrazepam (Dupont-NEN, specific activity 83.4 Ci/mmol), 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4) in a final reaction volume of 1 ml. 3 μM Clonazepam was used to define non-specific binding. Parallel reactions were done in the presence of 100 μM GABA, which maximally potentiates benzodiazepine binding (Gallager *et al.*, 1984). Samples were mixed by vortex, incubated in ice-water for 2 h and then, using a Brandel M-24R cell harvester, rapidly filtered through glass fiber filter paper (Whatman GF/B) and rinsed twice with 4 ml of ice cold 50 mM Tris-HCl (pH 7.4) buffer. The radioactivity retained on the filters was measured by liquid scintillation counting.

RESULTS

In membrane samples prepared from the cortex or cerebellum of control animals, 2 nM ^{3}H-flunitrazepam binding in the presence of 100 µM GABA was approximately 160% of that measured in the absence of GABA (figures 4.4 and 4.5). In response to a single dose of 15 mg/kg diazepam a marked, rapid reduction in the enhancement of ^{3}H-flunitrazepam binding by 100 µM GABA was observed in cortex and cerebellum (figure 4.4). Relative to the "0 hour" time point, this reduction reached significance at 4 hours post-injection (137.9%+/- 1.9% (3) and 144.0%+/-4.4% (3) (mean+/-SEM (n)) in cortex and cerebellum, respectively), was sustained at 12 hours post-injection (cortex, 137.4+/-2.9% (3); cerebellum, 142.7+/-0.7% (3)), and returned to baseline levels after 24 hours. Injection of 1 ml of sesame oil vehicle alone had no significant effect on GABA potentiation of benzodiazepine binding in cortex or cerebellum. Furthermore, 24 hours after 14 days of diazepam exposure there was no difference in GABA potentiation of benzodiazepine binding in treated versus control animals, in cortex or cerebellum (figure 4.5).

DISCUSSION

The most significant finding of the present study is a decrease in GABA potentiation of benzodiazepine binding in rat brain which can be seen as early as 4 hours after a single dose of diazepam. Care was taken to extensively wash membrane preparations and thereby avoid artifacts caused by residual diazepam. Potentially, diazepam remaining in membranes prepared from treated animals could displace ^{3}H-flunitrazepam and, because diazepam binding is enhanced by GABA to a greater degree than that of flunitrazepam (Doble and Martin, 1996), a decreased GABA shift could result. Minor variations in total ^{3}H-flunitrazepam binding which occurred in the present experiments were unrelated to the relatively large, progressive changes in GABA shift.

The rapidity of the decrease in GABA potentiation of benzodiazepine binding seen in the present study is consistent with reports of benzodiazepine-induced uncoupling in cell culture systems. Using primary cultures of chick neurons, Roca *et al.* (1990) showed a progressive decrease in GABA potentiation of flunitrazepam binding in response to continued flurazepam exposure, with a $T_{1/2}$ of approximately 18 hours. Heterologous expression of GABA$_A$ receptors in WSS-1 (Wong *et al.*, 1994) cells and Sf9 cells (Primus *et al.*, 1996), followed by exposure to several different benzodiazepine-site agonists, decreased GABA potentiation of ^{3}H-flunitrazepam binding on a similar timescale to that observed in cultured chick neurons. These decreases were sustained in the presence of the drug for the 48 to 60 hour duration of the experiments. In PA3 cells (Klein *et al.*, 1994; Klein *et al.*, 1995), which stably express a defined combination of GABA$_A$ receptor cDNA constructs under the control of a dexamethasone-sensitive promoter, a rapid ($T_{1/2}$= 32 min) sustained uncoupling effect was seen in response to benzodiazepine exposure. Interestingly, in PA3 cells, the presence of the non-specific protein kinase inhibitor staurosporine did not influence benzodiazepine-induced uncoupling, which suggests that this effect proceeds in the absence receptor phosphorylation. Furthermore, receptor internalization, as measured by differences in binding between a permeable and non-permeable benzodiazepine, was not detected during uncoupling. In cells transfected with GABA$_A$ receptor subunit cDNAs (Wong *et al.*, 1994; Klein *et al.*, 1994; Klein *et al.*, 1995; Primus *et al.*, 1996) receptor expression was not controlled by native GABA$_A$ receptor promoter elements. Thus, in these cells, it is unlikely that benzodiazepine-induced uncoupling was due to changes in receptor subunit composition. The fact that uncoupling was sustained in cultured cells but not in the present *in vivo* experiments could be explained in terms of drug metabolism. In cell culture the presence of drug is continuous, but in rat diazepam and its active metabolite N-desmethyldiazepam are each eliminated with a half-life of approximately one hour (Friedman et al., 1986). Thus, *in vivo*, allosteric coupling may be restored as drug is removed from the system by metabolism.

Previous studies have also investigated the regulation of allosteric coupling by benzodiazepines in whole animals. In cortical membranes prepared from rats treated for three weeks with either single daily injections of diazepam (Gallager *et al.*, 1984), or via implantation of continuous release silastic capsules (Hernandez *et al.*, 1989), reduced GABA potentiation of ^{3}H-flunitrazepam binding was observed. In cortical membranes derived from rats given flurazepam in their drinking water for 4 weeks, GABA potentiation of ^{3}H-flunitrazepam binding was similarly reduced (Tietz *et al.*, 1989). This effect was seen when the animals were sacrificed immediately, but not 48 hours after treatment, and there was no effect in rats intubated with diazepam 30 minutes before sacrifice. Thus, these experiments using whole animals indicated that *in vivo*, uncoupling was a chronic effect. In the present study, it has been shown that allosteric coupling changes significantly during the 24 hour period following a single dose of diazepam, but is unchanged in animals killed 24 hours after either a single or multiple daily diazepam injections. If there were a chronic component to the uncoupling effect *in vivo*, one would expect to see a greater degree of uncoupling in the animals sacrificed 24 hours after their last of 14 daily diazepam injections than in the animals sacrificed 24 hours following a single injection. This has, however, not been observed. Thus, considering the relatively rapid and sustained decrease in allosteric coupling seen in cultured cells, together with the results of the present study, it is likely that previously reported uncoupling effects in animals chronically exposed to benzodiazepines were actually a consequence of the acute action of the drug.

The results of the present study are inconsistent with a model of uncoupling based on altered GABA$_A$ receptor subunit composition. Data on the acute and chronic effects of diazepam exposure on GABA$_A$ receptor γ2-subunit gene expression in cortex and cerebellum are available, having been discussed in chapter three (see figures 3.1 and 3.2).

and data describing changes in α1-, β2- and γ2-subunit steady-state mRNA levels in cortex and cerebellum seen after chronic diazepam exposure are presented in figure 4.5. Thus, uncoupling is significantly reduced 4 hours after a single dose of diazepam and returns to baseline levels by 24 hours post-injection, but no changes in transcription of the $GABA_A$ receptor γ2 subunit, a subunit critically important for benzodiazepine modulation (Pritchett *et al.*, 1989a) are seen either 12 or 24 hours after administration of a single diazepam dose. In addition, whereas marked changes in the transcription and steady-state mRNA level (figures 3.1 and 4.6) of this subunit are observed following chronic diazepam exposure, there is no difference in allosteric coupling in rats killed 24 hours after either a single diazepam injection or multiple daily diazepam injections. Finally, and perhaps most importantly, diazepam-induced uncoupling proceeds in a very similar manner in cortex and cerebellum, whereas in these two brain regions diazepam has an inverse effect on the expression of the α1, β2 and γ2 subunits (figure 4.6), which comprise the most abundant $GABA_A$ receptor subtype (Mckernan and Whiting, 1996). While it is possible that uncoupling is mediated exclusively by a less abundant $GABA_A$ receptor subtype, the expression of which is regulated in the same direction in cortex and cerebellum, this seems unlikely considering the fact that uncoupling has been observed in $GABA_A$ receptors of variable subunit composition (Primus *et al.*, 1996).

It has previously been suggested that uncoupling is a means by which benzodiazepine tolerance develops (Gallager *et al.*, 1984). Evidence for a form of acute benzodiazepine tolerance, observed within hours of drug exposure, has been reported (Lister and Nutt, 1986; Crawford *et al.*, 1987) and may be related to the uncoupling phenomenon. However, as discussed in the introduction to chapter five, benzodiazepine dependence tends to increase in severity with the length of treatment and has been described in the vast majority of studies as a chronic rather than acute effect. Given the rapid onset and, *in vivo*, the short duration of uncoupling, plus the fact that it is not enhanced by chronic diazepam exposure, this phenomenon probably represents a form of acute tachyphylaxis rather than tolerance *per se*.

In conclusion, there is a rapid decrease in GABA potentiation of ^{3}H-flunitrazepam binding in cortical and cerebellar membranes prepared from rats which have received a single dose of diazepam. This effect does not appear to be enhanced by chronic treatment and it appears to be unrelated to diazepam induced-changes in GABA receptor gene expression. Uncoupling likely represents a fast post-transcriptional regulatory mechanism by which GABA$_A$ receptor molecular complexes respond to pharmacological stimulation.

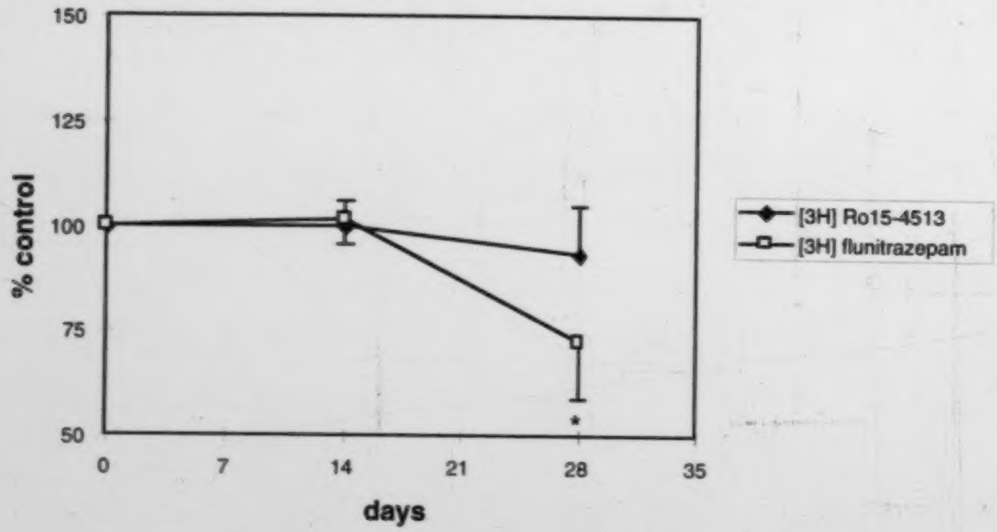

Figure 4.1. Total specific binding of ^{3}H-flunitrazepam and ^{3}H-Ro15-4513 in cortex after chronic diazepam exposure, expressed as a percentage of control values (+/- SEM). Statistical comparisons between treatment and control groups, at each time point, were done using unpaired, two-tailed Student's t tests (n=6, *P<0.05).

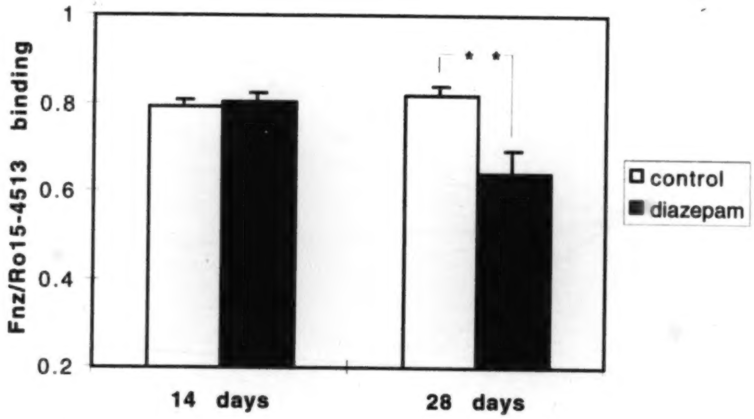

Figure 4.2. Ratios (+/- SEM) of ^{3}H-flunitrazepam to ^{3}H-Ro15-4513 binding in rat cortex after chronic diazepam exposure. Statistical comparisons between treatment and control groups, at each time point, were done using unpaired, two-tailed Student's t tests (n=6 in all but the 28 day control group, where n=12, **P<0.01).

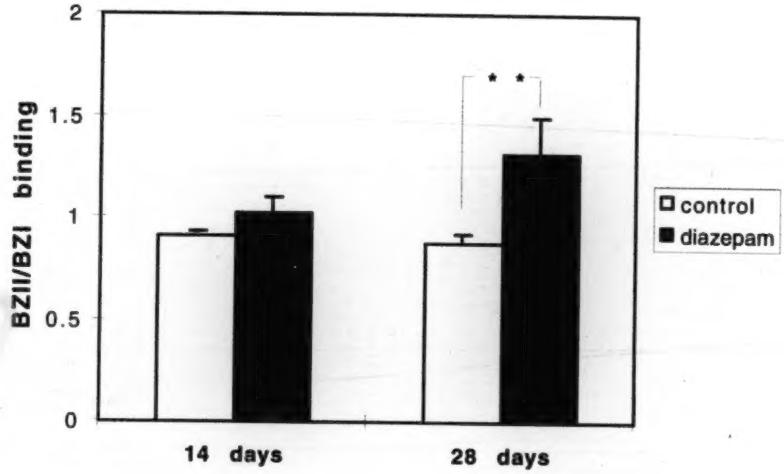

Figure 4.3. Ratios (+/- SEM) of type BZII to type BZI binding in rat cortex following chronic diazepam exposure. Statistical comparisons between treatment and control groups, at each time point, were done using unpaired, two-tailed Student's t tests (n=6 in all but the 28 day control group, where n=12, **P<0.01).

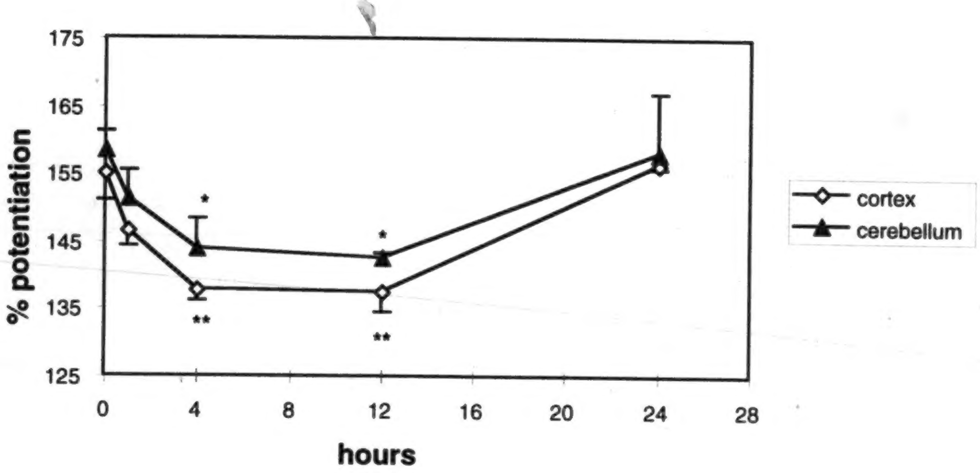

Figure 4.4. Potentiation of 2 nM ^{3}H-flunitrazepam binding by 100 μM GABA following a single dose of 15 mg/kg diazepam. Each data point is the mean of three experiments. The 0 through 12 hour time points were assayed together, and compared using Dunnett's multiple comparison test (*P<0.05, **P<0.01). The 24 hour time point was assayed later, in three separate sets of experiments, and a two-tailed unpaired Student's t-test indicated no significant difference from "0 hour" values.

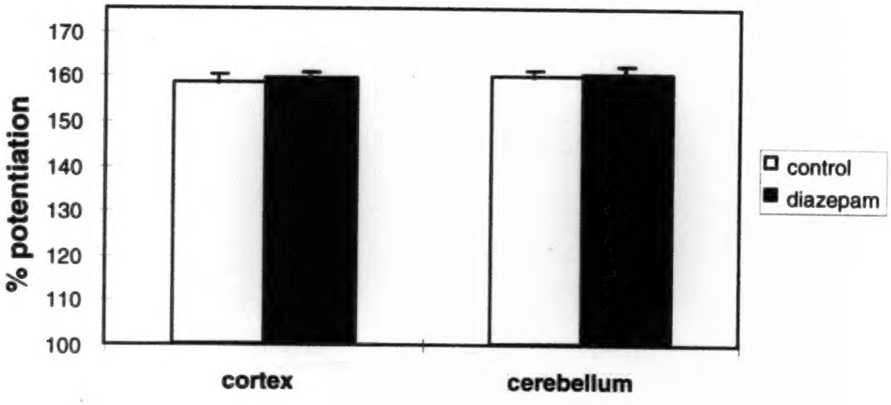

Figure 4.5. Potentiation of 2 nM ^{3}H-flunitrazepam binding by 100 μM GABA measured in rat cortex and cerebellum 24 hours following the last of 14 daily injections with 15 mg/kg diazepam. No differences were observed between the diazepam treatment and control groups, compared using a two-tailed unpaired Student's t-test.

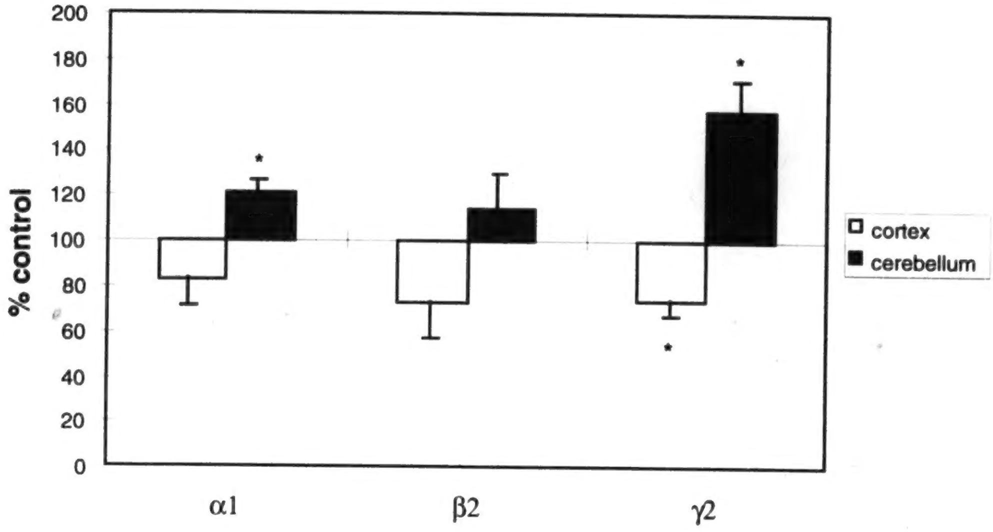

Figure 4.6. The effect of 14 daily 15 mg/kg doses of diazepam on $GABA_A$ receptor α1-, β2- and γ2-subunit steady-state mRNA levels in rat cortex and cerebellum quantified by solution hybridization, as described in the methods section, chapter two. These subunits are thought to comprise the most abundant $GABA_A$ receptor subtype. Data (mean +/- SEM, n=4-6) are expressed as a percentage of control values.

CHAPTER 5

The effect of abecarnil and zolpidem on $GABA_A$ receptor steady-state mRNA levels

Data presented in this chapter have been previously published. Holt, R.A., A.N. Bateson and I.L. Martin, 1996, Neuropharmacol. 35, 1457; Holt, R.A., A.N. Bateson and I.L. Martin, 1997, Eur. J. Pharmacol. 329, 129.

INTRODUCTION

Shortly after their introduction into clinical practice, it was observed that treatment with the benzodiazepines could lead to tolerance and physical dependence (Hollister et al., 1961). Tolerance is defined as a decrease in responsiveness to a drug as a consequence of continued administration, and physical dependence as a state produced by continued drug administration such that discontinuation causes a time-limited withdrawal reaction that can be reversed by resumption of treatment. The association of these phenomena with use of the classical benzodiazepines has been explored in detail through both clinical and animal studies (reviewed by Woods et al., 1987; Woods et al., 1992) and the results of these investigations support the following generalizations. Tolerance to the different therapeutic effects of the classical benzodiazepines seems to occur at different rates (Nutt, 1990; File, 1985) with the sedative effects being lost more rapidly than the anticonvulsant effects. The timecourse and degree of tolerance to the anxiolytic effects has been difficult to establish, which is perhaps a reflection of the inherent difficulty in quantifying small changes in anxiety levels in both patients and in animals. Regarding dependence, high doses of classical benzodiazepines are capable of producing this phenomenon in most subjects as evidenced by the appearance of withdrawal symptoms upon cessation of treatment. Rebound insomnia and rebound anxiety are the withdrawal effects most frequently seen clinically, and in animal studies muscle rigidity, enhanced locomotor activity and decreased seizure threshold are commonly measured endpoints. The benzodiazepines are also capable of producing dependence at normal therapeutic doses, but do so only in a proportion of subjects. The exact proportion of patients receiving therapeutic doses of classical benzodiazepines who are at risk for developing dependence is not clear (Woods et al., 1992), but it has been established in both clinical (Rickels et al., 1983; Rickels et al., 1990) and animal studies (Boisse et al., 1982; Rosenberg and Chui, 1985; Lukas and Griffiths, 1984; Boisse et al., 1990) that the risk and severity of withdrawal increases with dose and duration of treatment, with maximal dependence being reached after weeks to months of exposure. There is also evidence to suggest that withdrawal is more severe following treatment with short-acting versus long-acting benzodiazepines (Rickels et al., 1990; Busto et al., 1986) and that withdrawal severity can be reduced by gradually tapering the dose (Schweizer *et al.*, 1990).

The molecular mechanisms which underlie the development of benzodiazepine tolerance and physical dependence remain poorly understood. At moderate doses, these effects cannot be explained in terms of pharmacokinetics (Haigh et al., 1986; Miller et al., 1988), which is in agreement with the results of current experiments where drug concentrations measured in rat brain were unaffected by treatment duration. It is possible that the changes in $\mathrm{GABA_A}$ receptor gene expression that occur during chronic diazepam treatment, as described in chapter two, contribute to the development of benzodiazepine tolerance and physical dependence. In this chapter, the effect on $\mathrm{GABA_A}$ receptor gene expression of two benzodiazepine-site ligands with relatively low tolerance and dependence liability is investigated.

Abecarnil (a β-carboline) and zolpidem (an imidazopyridine) are benzodiazepine-site agonists which differ from the classical benzodiazepines in terms of subtype selectivity. Whereas the classical benzodiazepines have equally high affinity for $\alpha_x\beta_2\gamma_2$ recombinant $\mathrm{GABA_A}$ receptor constructs which contain either an $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$ subunit (Pritchett *et al.*, 1989b; Faure-Halley *et al.*, 1993) abecarnil has been reported to bind with six- and thirty-fold higher affinity to constructs containing the $\alpha1$ subunit than to constructs containing the $\alpha3$ and $\alpha5$ subunits, respectively (Pribilla *et al.*, 1993). In addition, abecarnil displays partial-agonism toward $\alpha2$ and $\alpha5$ subunit-containing receptors, but full agonism toward $\alpha1$ and $\alpha3$ subunit-containing receptors (Knoflach *et al.*,1993; Stephens *et al.*, 1991; Pribilla *et al.*, 1993). Zolpidem binds with high affinity to $\alpha1$ subunit-containing $\alpha_x\beta_2\gamma_2$ recombinant $\mathrm{GABA_A}$ receptor constructs, with approximately twenty-fold lower affinity to α2 and α3 subunit-containing constructs, and with negligible affinity to α5 subunit-containing constructs (Pritchett and Seeburg, 1990; Faure-Halley *et al.*, 1993).

Initial investigation of the anticonvulsant efficacy of abecarnil in dogs (Loscher, 1990) showed that over several weeks of treatment with this drug only a slight reduction in its ability to antagonize PTZ (pentylenetetrazole) induced seizures was observed in most animals, and in the remainder there was no reduction at all. Using electroencephalographic and electromyographic monitoring, and behavioral assessment of anxiety, Steppuhnn *et al.* (1993) subsequently showed that mice withdrawn from chronic abecarnil treatment did not display the time-related evolution of anxiety, muscle rigidity and seizures that was apparent after diazepam withdrawal. Serra *et al.* (1994) have shown that in contrast to diazepam, abecarnil is effective in inhibiting exploritory behavior in mice both before and after chronic administration. This group also found no evidence that chronic abecarnil treatment induced tolerance to isoniazid-induced seizures in normal rats (Serra *et al.*, 1995) or to PTZ-induced seizures in kindled (i.e. permanently PTZ-sensitized) rats (Serra *et al.* 1996). In a recent study, however, Loscher *et al.* (1996) present data that is not consistent with the view that abecarnil is devoid of tolerance and dependence effects. These experiments showed very similar reductions in motor impairment, sedation, and PTZ and electroshock seizure threshold upon chronic exposure to diazepam or abecarnil. In this study, these drugs were only notably different in that withdrawal of chronic diazepam was more likely to increase PTZ and electroshock sensitivity than withdrawal of chronic abecarnil.

Regarding zolpidem, no tolerance or withdrawal effects were seen in mice in association with chronic exposure, measured in terms of spontaneous motor activity or sensitivity to the convulsant effects of PTZ, electroshock or isoniazid (Perrault *et al.*, 1992). In contrast, Cox *et al.* (1988) reported that protection by zolpidem from PTZ-induced seizures was reduced after chronic treatment, but this reduction was modest in comparison with the decrease in seizure threshold seen after chronic diazepam treatment. In baboons, the ataxia and sedation produced by zolpidem was found to decrease over 1 week of administration, and the substitution of vehicle for zolpidem after chronic exposure caused a suppression of food intake, indicative of a drug withdrawal effect that is characteristic of termination of chronic diazepam (Griffiths *et al.*, 1992).

Thus, while there are some conflicting data regarding the magnitude of tolerance and dependence liability associated with abecarnil and zolpidem in treated animals, there is a body of evidence which suggest that the tolerance and withdrawal effects associated abecarnil and zolpidem treatment are less severe than those associated with exposure to diazepam. In the present study, steady-state $GABA_A$ receptor mRNA levels have been measured in rat brain following chronic exposure to abecarnil and zolpidem to determine if the effect of these drugs on $GABA_A$ receptor gene expression in any way contrasts with the effect of diazepam.

MATERIALS AND METHODS

Drug treatment

Male Sprague-Dawley rats (175 g to 200 g at initiation of treatment) were housed as described in chapter two. After habituation, rats were given subcutaneous, daily injections of 6 mg/kg abecarnil or 15 mg/kg zolpidem in 1 ml of sesame oil vehicle. All rats were killed by decapitation 2 hours after their final dose with the exception of the animals comprising the 14 day abecarnil treatment group, which were killed 24 hours after their last dose[2] . Immediately after sacrifice brains were dissected, frozen in liquid nitrogen and stored at -80°C.

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[2] The diazepam and abecarnil 14 day dosing regimens were the first to be completed. Because of difficulties in quantifying drug levels in these animals, which were sacrificed 24 hours after their final dose, animals in subsequent experiments were killed 2 hours after their final dose.

The results of the present study are directly comparable with the previously determined effects of 15 mg/kg of diazepam (described in chapter two) because *in vivo* binding studies have indicated that equivalent doses of zolpidem and diazepam, as used in the present experiments, give comparable levels of occupancy of the benzodiazepine binding site in rat cortex (Benavides *et al.*, 1992). Similarly, in mice, *in vivo* binding studies have indicated that 6 mg/kg abecarnil will give approximately the same level of receptor occupancy as 15 mg/kg diazepam (Steppuhn *et al.*,1993). In comparing the chronic effects of different agents it is important to use equivalent doses, but it is also preferable for the kinetics of the drugs being investigated to be equivalent. In the rat, the elimination half-lives of abecarnil (1.7 h; Krause *et al.*, 1990) and zolpidem (1.5 h; Garrigou-Gadenne *et al.*, 1989) are very similar to the elimination half-lives of diazepam and its primary active metabolite N-desmethyl diazepam (each approximately 1.0 h; Klotz *et al.*, 1976; Friedman *et al.*, 1986).

Quantification of CNS drug levels by reversed-phase HPLC

Abecarnil:

100 mg of cortex or spinal cord was taken from each animal treated with abecarnil, homogenized in five volumes of methanol and centrifuged 15 minutes at 12000 x g. A 50μl aliquot of the supernatant was injected onto a 30 cm Spherisorb C18 5 μm column (Alltech) and eluted at a flow rate of 1.5 ml/min using a solvent gradient that increased linearly from 65% A:35% B (v/v) to 85% A:15% B (v/v) in 12 minutes, with "A" being acetonitrile and "B" being 13 mM sodium phosphate buffer (pH 7). Peaks were detected using a fluorescence detector with an excitation wavelength of 295 nm and an emission wavelength of 418 nm. The β-carboline ZK91296 (4 ng per 50 μl injected) was used as an internal standard.

For each assay a standard curve was generated (as described in chapter 2) using 50 µl samples of blank brain extract containing 4 ng of ZK91296 and either 0.5, 1, 2 or 5 ng of abecarnil. By comparison to the standard curve, which was linear over the range of abecarnil concentrations used, the amount of abecarnil present in extract prepared from each drug-treated animal was determined.

Zolpidem:

100 mg of cortex was taken from each animal treated with zolpidem, homogenized in five volumes of methanol and centrifuged 15 min at 12000 x g. A 50 µl aliquot of the supernatant was injected onto a 30 cm Spherisorb C18 5 µm column (Alltech) and eluted isocratically with 70% (v/v) acetonitrile:tetrahydrofuran (10:1) and 30% (v/v) 13 mM sodium phosphate buffer (pH 7) mobile phase at a flow rate of 1 ml/min. Peaks were detected using a fluorescence detector with an excitation wavelength of 254 nm and an emission wavelength of 390 nm. Harmane (4 ng per 50 µl injected) was used as an internal standard.

For each assay a standard curve was generated (as described in chapter 2) using 50 µl samples of blank brain extract containing 4 ng of harmane and either 0.05, 0.1, 0.5 or 1ng of zolpidem. By comparison to the standard curve, which was linear over the range of zolpidem concentrations used, the amount of zolpidem present in extract prepared from each drug-treated animal was determined.

Quantification of GABA$_A$ receptor steady-state mRNA levels by solution hybridization

The quantification of GABA$_A$ receptor steady-state mRNA levels was performed as described in the methods section, chapter two.

RESULTS

Abecarnil and zolpidem concentrations in rat brain following chronic exposure

The mean concentrations of abecarnil in rats killed 2 hours after their last of 7 or 28 daily injections were 110.6+/-9.8 (6) (mean+/-SEM, (n)) and 109.1+/-16.1 (4) nanograms per gram of cortex, respectively, and the mean concentrations of zolpidem in rats killed 2 hours after their last of 7 or 14 daily injections with zolpidem were 181.0+/-12.6 (6) and 200.5+/-19.6 (6) nanograms per gram of cortex, respectively. There were no significant differences between groups treated with the same drug for different lengths of time.

The effects of abecarnil and zolpidem on GABA$_A$ receptor subunit mRNA levels

The time points and brain regions examined following abecarnil treatment were the same as for the experiments involving diazepam (chapter two). Results are shown in table 5.1. The levels of γ2- and β2-subunit mRNA were significantly reduced in cortex and significantly increased in hippocampus following 14 days of abecarnil exposure. After 28 days of abecarnil treatment α4- and β1-subunit mRNA levels were significantly increased in hippocampus. No other significant changes were observed.

The effect of zolpidem on GABA$_A$ receptor subunit mRNA levels was examined only in cortex, following 7 days or 14 days of exposure, as shown in table 5.2. The levels of α4- and β1-subunit mRNAs were significantly increased after 7 days of zolpidem treatment and, following two weeks of treatment, a significant decrease in the level of α1-subunit mRNA was observed. Neither one nor two weeks of zolpidem treatment produced any significant effect on the amount of any other GABA$_A$ receptor subunit mRNA in rat cortex. As noted in chapter two, treatment with sesame oil vehicle alone did not significantly alter the level of any of the $GABA_A$ receptor subunit mRNA species in cortex or hippocampus at any of the time points examined. The abundance of γ1- and γ3-subunit mRNA in hippocampus was too low to allow quantification and, in both the cortex and hippocampus, the level of α6-subunit mRNA was not sufficient for quantification.

DISCUSSION

The relative steady-state levels of different $GABA_A$ receptor subunit mRNAs in rat brain following chronic abecarnil and zolpidem treatment have been determined. This is the only study that has investigated the effects of these drugs on $GABA_A$ receptor gene expression, and in comparison with the effects of diazepam, several interesting features in the data become apparent. The striking similarity between the effects of diazepam and abecarnil in hippocampus is most obvious. The only abecarnil-induced changes in hippocampus (an increase in γ2- and β2-subunit mRNA levels after 14 days exposure, and α4- and β1- subunit mRNA levels after 28 days of exposure) are precisely the same changes which occur in response to diazepam. In hippocampus, the only apparent differences in the effects of these two drugs are the magnitude of the increase in γ2-subunit mRNA level after 7 days treatment and the magnitude of the rebound in γ2- and β2-subunit mRNA levels following withdrawal, which are greater in each case in the diazepam group. These results clearly indicate that the high dependence potential of diazepam cannot be explained in terms of selective changes in $GABA_A$ receptor subunit mRNA levels in hippocampus.

In cortex there were several similarities but also several differences in the effects of diazepam and abecarnil on $GABA_A$ receptor mRNA levels. No changes in cortical mRNA levels were detected in either treatment group following 28 days of treatment or after 9 days of withdrawal. However, during the first two weeks of exposure, diazepam and abecarnil both caused a reduction in the levels of γ2-subunit mRNA, and there was a decrease in β2-subunit mRNA levels which reached significance in the abecarnil treatment group only. Of particular interest, diazepam treatment caused large and significant increases in the levels of α3-, α4-, α5-, β1- and γ3- subunit mRNA levels within the first two weeks of treatment -- changes which were not observed in the abecarnil treatment groups.

To obtain additional data on the degree to which these changes in cortex are diazepam specific, $GABA_A$ receptor mRNA levels were measured in the cortex of rats treated for one or two weeks with zolpidem. It was found that zolpidem had a similar effect to diazepam on α4 and β1- subunit mRNA levels in rat cortex, within the first two weeks of treatment. However, zolpidem, like abecarnil, failed to increase α3-, α5- and γ3-subunit mRNA levels. Therefore, the only consistent difference in the profile of mRNA changes between diazepam and the drugs with lower dependence potential (abecarnil and zolpidem) appears to be the diazepam-specific effect on α3-, α5- and γ3-subunit mRNA levels in cortex, which occurs within the first two weeks of exposure (figure 5.1). Predictions from recombinant receptor studies concerning the consequences of changes in the expression of these subunits will be discussed in chapter six (general discussion). Of note, an association between diazepam-mediated increases in α5 subunit mRNA and diazepam tolerance has also been shown by Impagnatiello *et al.* (1996). This group compared steady-state $GABA_A$ receptor mRNA levels in selected neocortical regions of rats chronically treated with either diazepam or the benzodiazepine-site partial agonist imidazenil. Diazepam tolerant rats showed a significant increase in the level of α5-subunit mRNA in the frontoparietal motor cortex while the level of α5-subunit mRNA in imidazenil treated animals, which failed to develop tolerance, was unchanged. This group did not report any significant changes in α3-subunit mRNA levels, and they did not examine the level of γ3-subunit mRNA.

Diazepam like other classical benzodiazepine agonists has an extensive therapeutic profile; while it is used principally in the treatment of anxiety and sleep disorders, this drug also has muscle relaxant and anticonvulsant activity. It is not known, however, which brain regions or neurological pathways mediate the different effects of diazepam. The present results are consistent with the idea that the changes in expression of the α3-, α5- and γ3-subunit genes may contribute to tolerance to those effects of diazepam which are mediated by its action in the cortex. Likewise, changes in the expression of these subunit genes may be the cause of diazepam withdrawal reactions which are cortically mediated.

As discussed in the introduction to this chapter many studies have found no evidence for tolerance or withdrawal effects following the chronic administration of abecarnil or zolpidem, but there are some studies which have. The fact that tolerance and dependence effects are not consistently observed in the laboratory in association abecarnil and zolpidem treatment suggests that the tolerance and dependence liabilities of these drugs, while not completely absent, are lower than for diazepam, which has a more robust effect experimentally. The results of the present study are consistent with this interpretation. That is, $GABA_A$ receptor mRNA induction by abecarnil or zolpidem may underlie the limited degree of tolerance and dependence associated with these drugs and diazepam's tolerance and dependence liability may be greater due to its extended effect on $GABA_A$ receptor mRNA induction.

As a final observation, the possibility of a mechanism of coordinate control over drug-induced changes in expression of $GABA_A$ receptor genes (which tend to be organized as $\alpha/\beta/\gamma$ clusters) was discussed in chapter two, and the data on abecarnil and zolpidem presented in this chapter are consistent with this premise (figure 5.3). Considering the fact that two of the three genes located within the chromosome 15 cluster (the $\alpha5$ and $\gamma3$ subunit genes) are specifically modulated by diazepam, this particular cluster is a prime candidate for a genetic locus of benzodiazepine tolerance and dependence.

Table 5.1. The effect of chronic abecarnil treatment and withdrawal on $GABA_A$ receptor subunit steady-state mRNA levels in rat cortex and hippocampus. The mean ratios of $GABA_A$ receptor subunit band intensities to β-actin band intensities are expressed as a percentage of untreated control values (+/- SEM). At each time point, different drug treatment groups (diazepam, abecarnil or zolpidem) were typically assayed together. Statistical comparisons were carried out between drug treatment and control groups using one way analysis of variance followed, if significance was achieved, by Newman Keuls test for multiple comparisons.

(n=3-6, *P<0.05).

Cortex

subunit	7 days	14 days	28 days	28 days & withdrawal
α1	101.1 +/- 11.6	97.6 +/- 6.4	103.2 +/- 11.4	106.8 +/- 8.3
α2	87.4 +/- 8.7	113.6 +/- 6.5	136.4 +/- 5.8	100.5 +/- 10.9
α3	96.0 +/- 10.9	108.8 +/- 17.2	101.6 +/- 10.5	83.4 +/- 11.2
α4	82.3 +/- 16.6	88.9 +/- 7.4	94.3 +/- 14.1	100.7 +/- 7.5
α5	105.6 +/- 14.5	109.9 +/- 13.3	102.0 +/- 20.4	94.2 +/- 3.3
β1	109.8 +/- 10.7	92.8 +/- 7.4	90.3 +/- 15.8	94.0 +/- 2.3
β2	76.9 +/- 12.1	*73.3 +/- 4.8	78.5 +/- 12.1	131.0 +/- 5.3
β3	90.0 +/- 12.0	101.2 +/- 28.8	101.0 +/- 8.8	99.9 +/- 2.1
γ1	112.7 +/- 16.6	87.9 +/- 23.4	101.3 +/- 25.3	91.3 +/- 4.6
γ2	79.7 +/- 9.8	*59.1 +/- 7.7	86.9 +/- 9.0	123.5 +/- 6.5
γ3	98.3 +/- 22.8	102.1 +/- 7.3	83.0 +/- 17.6	80.3 +/- 10.5

Hippocampus

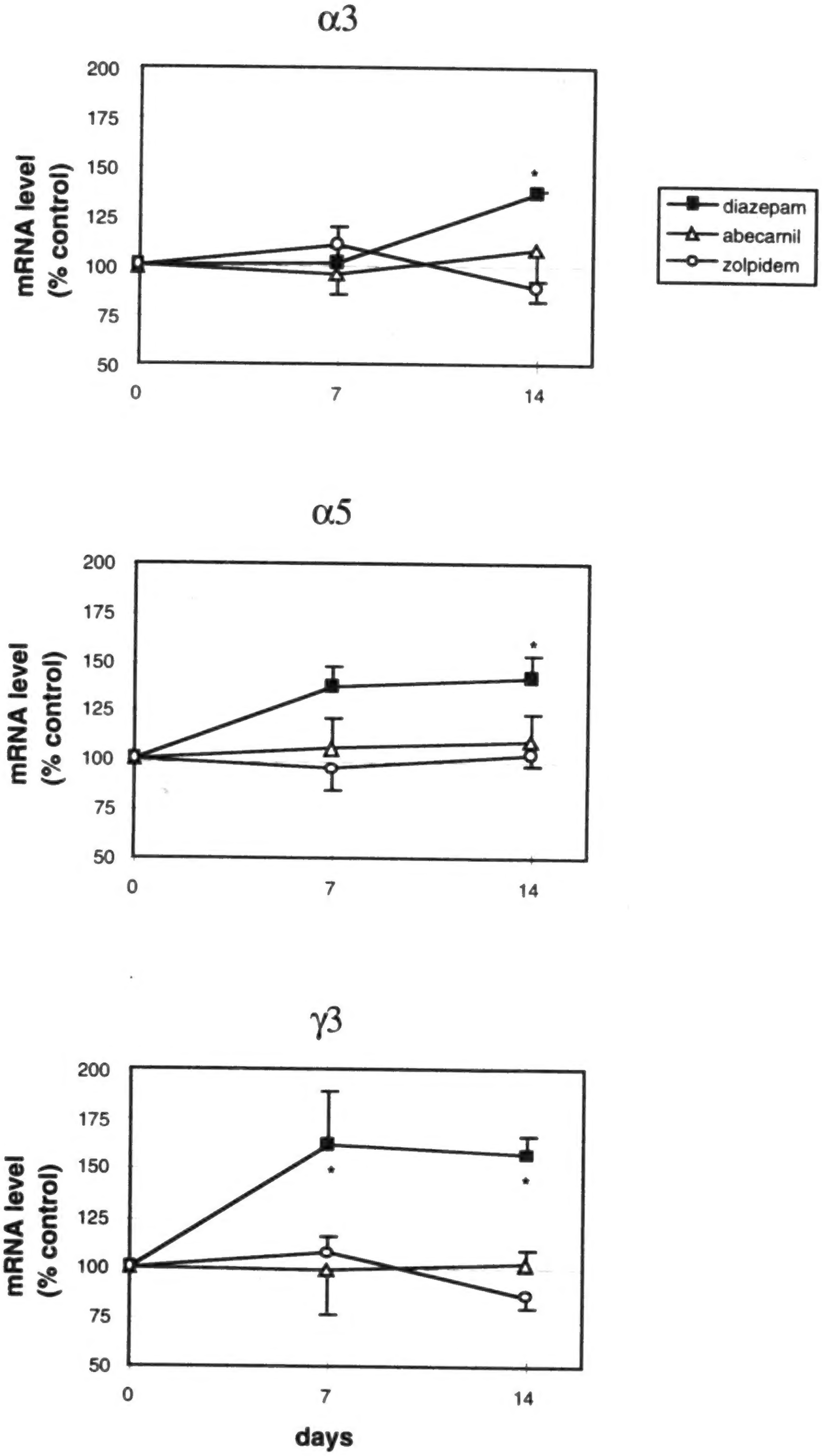
subunit	7 days	14 days	28 days	28 days & withdrawal
α1	86.7 +/- 5.7	116.9 +/- 11.2	132.5 +/- 31.6	74.6 +/- 9.2
α2	101.3 +/- 7.7	105.6 +/- 4.8	110.9 +/- 11.8	72.3 +/- 17.7
α3	128.1 +/- 17.6	113.4 +/- 4.2	86.1 +/- 16.6	61.7 +/- 9.7
α4	144.9 +/- 11.7	115.0 +/- 21.8	*136.6 +/- 8.7	89.7 +/- 21.2
α5	100.1 +/- 10.8	97.9 +/- 7.9	112.8 +/- 1.1	90.0 +/- 4.1
β1	100.2 +/- 10.8	111.8 +/- 8.3	*148.7 +/- 9.5	54.2 +/- 22.2
β2	93.5 +/- 16.0	*144.8 +/- 7.2	121.8 +/- 26.1	94.3 +/- 10.1
β3	100.2 +/- 13.8	102.7 +/- 7.7	94.2 +/- 3.3	103.1+/- 7.1
γ2	115.9 +/- 11.6	*147.8 +/- 5.1	100.1 +/- 12.6	61.2 +/- 26.4

Cortex

subunit	7 days	14 days
α1	94.6 +/- 11.0	*72.7 +/- 11.1
α2	125.7 +/- 12.9	111.0 +/- 8.9
α3	110.9 +/- 11.6	88.6 +/- 15.0
α4	*170.8 +/- 10.3	110.1 +/- 9.8
α5	95.2 +/- 11.3	102.0 +/- 4.3
β1	*149.1 +/- 5.5	118.7 +/- 6.5
β2	99.9 +/- 9.4	92.4 +/- 7.4
β3	90.0 +/- 23.0	100.5 +/- 13.3
γ1	116.5 +/- 11.2	120.1 +/- 7.6
γ2	95.1 +/- 3.5	86.1 +/- 5.3
γ3	106.6 +/- 8.9	84.8 +/- 6.2

Table 5.2. The effect of chronic zolpidem treatment on $GABA_A$ receptor subunit steady-state mRNA levels in rat cortex. The mean ratios of $GABA_A$ receptor subunit band intensities to β-actin band intensities are expressed as a percentage of untreated control values (+/- SEM). At each time point different drug treatment groups (diazepam, abecarnil or zolpidem) were typically assayed together. Statistical comparisons were carried out between drug treatment and control groups using one way analysis of variance followed, if significance was achieved, by Newman Keuls test for multiple comparisons.
(n=4-6, *$P<0.05$).

Figure 5.1. Differential effects of effects chronic diazepam, abecarnil, and zolpidem treatment on $GABA_A$ receptor $\alpha3$-, $\alpha5$- and $\gamma3$-subunit steady-state mRNA levels in rat cortex, expressed as percentages of control values. $^*P<0.05$, indicates a significance difference in comparison to control. Data are taken from tables 2.2, 5.1 and 5.2.


α3
α5
γ3
mRNA level (% control)
200
175
150
125
100
75
50
0
7
14
days
*
diazepam
abecarnil
zolpidem

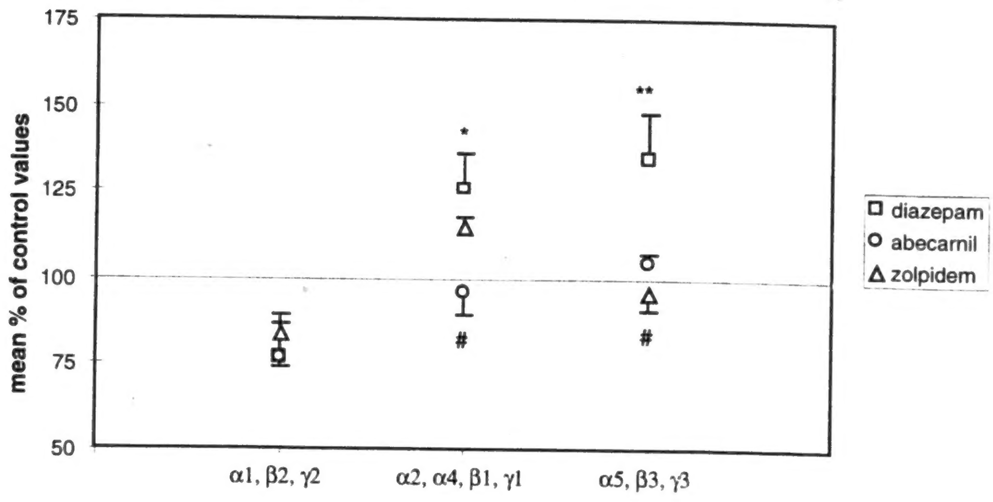


Figure 5.3. Association between the mean steady-state mRNA level of clustered $GABA_A$ receptor genes in rat cortex following chronic diazepam, abecarnil and zolpidem exposure. To compare the effects of diazepam, abecarnil and zolpidem on coordinate expression of $GABA_A$ receptor gene expression, steady-state mRNA levels measured after 14 days of treatment with each of these three drugs have been grouped according to human gene cluster and the mean mRNA level of each cluster (+/- SEM) has been plotted. Statistical comparisons have been carried out using Newman Keuls test.

** Indicates a significant difference in comparison to the effect of chronic diazepam on expression of the α1–, β2– and γ2-subunit clustered genes, $P<0.01$.

* Indicates a significant difference in comparison to the effect of chronic diazepam on expression of the α1–, β2– and γ2-subunit clustered genes, $P<0.05$.

# Indicates as significant difference in comparison with the effect of chronic diazepam on the expression of the same cluster of genes, $P<0.05$.

CHAPTER 6

General discussion

The multimeric $GABA_A$ receptor is a molecule of central importance in both a biological context, being the principal mediator of the actions of the most abundant and widely distributed neurotransmitter in the brain, GABA, and in a therapeutic context, being the molecular target of a wide variety of pharmacological agents used in the treatment of diverse central nervous system disorders. The aim of this dissertation has been to improve our understanding of how extended treatment with drugs that act via the $GABA_A$ receptor can affect its properties. Because therapeutic drugs, like other xenobiotics, present an environmental insult to an organism, homeostatic responses are typically seen in association with chronic exposure and may underlie drug tolerance and physical dependence. The specific working hypothesis maintained throughout this dissertation has been that chronic exposure to benzodiazepines, drugs which are effective in the treatment of anxiety and sleep disorders because of their ability to modulate $GABA_A$ receptor function, changes the expression of particular $GABA_A$ receptor subunit genes. *In vitro* expression of recombinant $GABA_A$ receptors has shown that the presence of different subunits within receptor constructs can confer different functional and ligand recognition properties. Thus, through regulating the abundance of $GABA_A$ receptors containing particular subunits, the brain has a unique opportunity to protect itself from the presence of these drugs.

The results of experiments described in this dissertation are largely consistent with the above hypothesis, which they were designed to test. In chapter two, it was shown that chronic exposure to the quintessential benzodiazepine diazepam alters the steady-state mRNA level of selected $GABA_A$ receptor subunit isoforms in a time- and brain region-specific manner. Chapter three indicated that these changes potentially involve modification of $GABA_A$ receptor gene transcription, and in chapter four has it was shown that observed changes in steady-state mRNA levels are consistent with diazepam-induced changes in receptor protein, characterized in terms of the recognition properties of the benzodiazepine binding site.

In rat cortex, the brain region principally investigated in these studies, changes in $GABA_A$ receptor subunit expression caused by diazepam included a significant increase in the level of α3-, α4-, α5-, β1- and γ3-subunit mRNA and a significant decrease in the level of γ2-subunit mRNA. Predictions regarding the consequences of these changes based on the results of recombinant receptor studies are made below and, overall, they would be expected to confer reduced diazepam sensitivity. However, it must be stressed that extrapolating the properties of recombinant receptors to native receptors presents some difficulties (for example, the properties conferred by a given subunit can vary depending upon which other subunits are present, and the subunit composition of native $GABA_A$ receptors is undefined). Thus the following predictions are necessarily hypothetical in nature. The largest diazepam-induced change in the steady-state level (relative to control) of an mRNA species was that sustained by the α4-subunit mRNA species. As the presence of the α4 subunit in $\alpha_x\beta_2\gamma_2$ recombinant receptors abolishes affinity for most benzodiazepine-site ligands, including diazepam (Wisden *et al.*, 1991), the predicted consequence of diazepam-induced increase in α4-subunit mRNA is attenuation of the action of this drug. Likewise, the diazepam-induced increase in γ3-subunit mRNA and decrease in γ2-subunit mRNA would be expected to reduce diazepam's modulatory action as the presence of the γ3 subunit in $\alpha_1\beta_2\gamma_x$ recombinant receptors confers low affinity, and the presence of the γ2 subunit confers high affinity, for benzodiazepine-site agonists (Pritchett *et al.*, 1989a; Herb *et al.*, 1992). In terms of receptor binding it is not clear how diazepam-specific increases in α3 or α5 subunit-containing receptors are related to reduced diazepam sensitivity as the affinity of diazepam for recombinant receptors containing either of these subunits is approximately equal to its affinity for α1 or α2 subunit-containing recombinant receptors (Pritchett *et al.*, 1989b; Faure-Halley *et al.*, 1993). It has, however, been reported that the ability of diazepam to potentiate GABA-gated chloride currents in recombinant receptors is lower in those which contain the α5 subunit than in those containing an α1, α2, or α3 subunit (Puia et al., 1991) which may provide some rationale for the induction of α5-subunit mRNA. As mentioned in the general introduction, the exchange of one β subunit for another in recombinant receptor constructs does not seem to alter sensitivity to benzodiazepine-site ligands (Hadingham *et al.*, 1993), therefore no predictions regarding consequences of the increase in β1-subunit mRNA level can be made at this time.

The majority of the present work has focused on the effects of chronic diazepam on $GABA_A$ receptors specifically in rat cortex. However, some experiments have been conducted using other brain areas and have revealed interesting regional differences in the effect of diazepam on $GABA_A$ receptor gene expression. Most notably, expression of the γ2-subunit gene is modulated in an opposite direction in hippocampus and cerebellum than in cortex, and there is evidence that this effect extends to modulation of the α1- and β2-subunit genes, which are present in the same gene cluster (see figures 2.6 and 4.6). The physiological basis for the inverse nature of this effect is unknown, but may be related to either different mechanisms of transcriptional control operating in cortical versus hippocampal or cerebellar neurons, or different neuronal circuitry in these brain regions. The possibility that $GABA_A$ receptor gene expression is sensitive to the overall effect of diazepam on the activity of GABAergic circuits and not just to its action on individual cells is supported by the observation that, in spite of reports that it has no affinity for α4 subunit-containing recombinant receptors, diazepam can affect α4-subunit gene expression.

It can not be concluded from the present experiments that diazepam causes $GABA_A$ receptor subtype switching specifically within individual cells, as the effects of this drug have been investigated using whole anatomical regions of rat brain. The interpretation that diazepam exposure has differential effects on specific types of neurons, but for any given cell modulates only those receptor subtypes that are constituatively expressed, is consistent with the current data. To specifically test this possibility it would be necessary to use as an experimental system a homogenous population of cells which reliably express a defined set of GABA$_A$ receptor subunits under the control of their native promoters. Unfortunately, an experimental system that meets these criteria is not currently available.

It is possible that the neurochemical effects of diazepam described in this dissertation are associated with the behavioral phenomena of benzodiazepine tolerance and dependence. As discussed in the introduction to chapter five, evidence in the literature suggests that diazepam has higher dependence and tolerance liability than either abecarnil or zolpidem, although there is some disagreement between studies. Interestingly, in the present study the pattern of diazepam-induced changes in GABA$_A$ receptor steady-state mRNA levels was different than the pattern of changes produced by abecarnil and zolpidem. Diazepam, but not abecarnil or zolpidem, significantly altered the steady-state mRNA levels of the $\alpha3$, $\alpha5$ and $\gamma3$ subunits in rat cortex (figure 5.1). Considering the prediction, made above, that increased expression of the $\alpha5$ and $\gamma3$ subunit genes could confer diazepam insensitivity, this observation provides some neurological basis for differences between these drugs in terms of tolerance and dependence liability. However, it must be stressed that there are effects on GABA$_A$ receptor subunit mRNA levels that are seen in response to all three of these drugs. For example, diazepam, abecarnil and zolpidem all increase at some point the level $\alpha4$-subunit mRNA, an effect which would be expected in each case to confer insensitivity to the drug of treatment. Generally speaking, however, the illustration that these drugs can differentially modulate GABA$_A$ receptor steady-state mRNA levels is consistent with the idea that these sorts of neurochemical effects are related to tolerance and dependence.

In conclusion, the studies presented here have demonstrated that chronic treatment of rats with diazepam (1) alters GABA$_A$ receptor steady-state mRNA levels in a manner which is time- and brain-region specific and which could confer diazepam insensitivity, (2) alters transcription of the GABA$_A$ receptor $\gamma2$-subunit gene in a manner that is consistent with the effect of this drug on $\gamma2$-subunit steady-state mRNA levels and (3) alters the benzodiazepine-site binding characteristics of native GABA$_A$ receptors in a manner that is consistent with the effects of this drug on $GABA_A$ receptor steady-state mRNA levels. These findings are consistent with the hypothesis that benzodiazepine exposure evokes subtype-specific changes the population of $GABA_A$ receptors in rat brain as a protective response. As diazepam-induced changes on $GABA_A$ receptor steady-state mRNA are in some ways distinct from those induced by abecarnil and zolpidem, it is possible that these effects are related to benzodiazepine tolerance and physical dependence.

Future directions

A collaboration between our laboratory and a laboratory in the UK that has expertise in the behavioral effects of benzodiazepines has been arranged, and experiments are planned which will compare, in rats, the timecourse of tolerance development to various behavioral effects of diazepam with the timecourse of diazepam-induced changes in $GABA_A$ receptor steady-state mRNA levels. The results of these experiments should help clarify the relationship between benzodiazepine-induced changes in the expression of $GABA_A$ receptor genes and the behavioral manifestations of benzodiazepine tolerance.

An interesting trend observed in the results of the present experiments was an association between the responses of clustered $GABA_A$ receptor genes to benzodiazepine-site agonist exposure (see figures 2.6 and 5.2). While evidence for this association is purely observational at this point, it would be interesting to know its molecular basis and functional relevance. As noted in chapter five, two of the three $GABA_A$ receptor genes modulated by diazepam, but not abecarnil or zolpidem (the $\alpha5$- and $\gamma3$- subunit genes), are present in the same gene cluster. The third (the $\alpha3$-subunit gene) is not associated with a $GABA_A$ receptor gene cluster. The possibility that the cluster to which the $\alpha5$- and $\gamma3$-subunit genes belong is a genetic locus of benzodiazepine tolerance and dependence can be specifically tested. In mice, a particular mutation (the pink-eyed cleft-palate mutation) is associated with deletion of the $GABA_A$ receptor $\alpha5$-, $\gamma3$- and part of the $\beta3$-subunit gene (Nakatsu *et al.,* 1993). Homozygous mutant mice show hypopigmentation, cleft palate and certain neurological deficits including tremor and a jerking gait. If the behavioral characteristics of these mice remain intact to the extent that behavioral responses to benzodiazepine exposure can be measured, then reduced tolerance or withdrawal effects in these animals would provide evidence that the $\alpha5/\beta3/\gamma3$ subunit gene cluster is involved in mediating benzodiazepine tolerance and dependence.

Finally, while it is clear that diazepam can modulate $GABA_A$ receptor steady state mRNA levels, the molecular mechanism by which this effect occurs is unknown. An important step in elucidating this mechanism has been made in the present study, where it has been shown that diazepam can modulate the expression of a $GABA_A$ receptor gene specifically at the level of transcription. The nature of the nuclear signal through which diazepam elicits this effect is unknown, but the signaling pathway probably begins at the cell surface with changes in the functional state of the receptor. Accordingly, exposure to other $GABA_A$ receptor modulatory agents such as ethanol (Montpied et al., 1991a; Mhatre and Ticku, 1992) pentobarbital (Tseng et al., 1993) or to GABA (Montpied et al., 1991b) can also affect $GABA_A$ receptor steady-state mRNA levels. One can speculate that the signal generated in response to receptor activity takes the form of some second messenger molecule or, alternatively, perhaps the control of $GABA_A$ receptor gene expression is an auto-regulatory process. Receptor internalized from the cell surface may directly modulate gene expression via translocation (of the holo-receptor or subunits or fragments thereof) to the nucleus and interaction with regulatory elements in target genes or with other components of the transcriptional machinery.

A rational approach to elucidating the mechanism by which diazepam or other allosteric modulators regulate $GABA_A$ receptor gene expression is to establish how these genes are regulated under normal physiological conditions, then explore the effects of drug exposure. Taking this approach, preliminary studies in our laboratory have revealed a pattern of DNase hypersensitivity sites (i.e. putative sites of regulatory factor interaction) in the promoter of the $GABA_A$ receptor $\alpha 1$-subunit gene in rat brain that are associated with regions which confer repressor- and enhancer-like activity in functional assays. Further investigations of this nature are planned and these will explore in comprehensive manner the mechanisms controlling $GABA_A$ receptor gene expression. The molecular basis of the effect of benzodiazepines on $GABA_A$ receptor gene expression will, in time, be revealed, as may novel targets for pharmacologically manipulating GABAergic transmission.

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